UNRAVELING THE COMPLEXITIES OF IDIOPATHIC PAIN DISORDERS: FROM MOUSE TO MAN

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ABSTRACT

Brittney Pauline Ciszek: Unraveling the Complexities of Idiopathic Pain Disorders: From Mouse to Man
(Under the direction of Andrea G. Nackley)

Idiopathic pain disorders (IPDs) are common in the population, yet poorly understood and managed. It is generally accepted that IPDs result from variability in genetic and environmental factors that impact the expression of pain-relevant genes, such as catechol-O-methyltransferase (COMT; an enzyme that metabolizes catecholamines). Human genetic variants coding for reduced levels of COMT are associated with increased experimental pain and IPD risk. Preclinical work in our lab has further shown that COMT-dependent pain is mediated *via* β_2- and β_3-adrenergic receptors (ARs) and downstream signaling mediators. Emerging evidence also implicates a role for microRNAs, regulators of gene expression, in IPDs and adrenergic signaling. Yet, more work is required to elucidate the contribution of distinct populations of βARs and microRNAs to persistent pain in preclinical and clinical settings. Thus, the present studies aim to 1) determine which βAR-enriched tissues drive COMT-dependent pain and 2) identify specific microRNAs associated with COMT-dependent and idiopathic pain. To identify a site-of-action for COMT-dependent pain, we measured the ability of peripheral, spinal, and supraspinal β_2- and β_3-AR antagonists to block the development of pain initiated by a COMT inhibitor and to reverse pain established in COMT-/- mice. Peripheral antagonist administration blocked pain produced by COMT pharmacologic inhibition, but not gene knockdown. This suggests that peripherally-located β_2- and β_3-ARs initiate, but do not maintain COMT-dependent pain. Next, to elucidate the role of microRNAs in persistent pain, we examined whole blood microRNA expression profiles collected from rats receiving the COMT inhibitor or from patients with IPDs. Results

demonstrated that COMT-dependent pain and IPDs are each associated with significant microRNA dysregulation. Clinical results further demonstrated that microRNA expression profiles differentiate IPD patients into two subtypes with distinct pain phenotypes, psychological characteristics, and biological correlates. Collectively, the results presented in these studies suggest that βARs and microRNAs represent novel targets for the treatment of idiopathic pain. Peripherally-acting βAR antagonists may be beneficial in acute clinical settings to prevent the development of idiopathic pain among susceptible individuals. MicroRNAs and/or microRNA inhibitors may also represent novel treatments for established pain as well as to diagnose and differentiate IPD subtypes.

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“One of the problems of opening yourself to gratitude is that the big things have no words. There is only being there in the wonder of it, not knowing if you’ll ever speak again…”
- Brian Andreas

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α	alpha
ADCY9	adenylate cyclase type 9
AdoMet	S-adenosyl-L-methionine
Adx	adrenalectomized
AKT1	protein kinase B
ANCOVA	analysis of covariance
ANOVA	analysis of variance
AR	adrenergic receptor
β	beta
βAR	β-adrenergic receptor
BDNF	brain-derived neurotrophic factor
BL	baseline
BPS	bladder pain syndrome
cAMP-PKA	cyclic adenosine monophosphate kinase
CCL2	chemokine C-C motif ligand 2
CFA	Complete Freund’s Adjuvant
CIP	congenital insensitivity to pain
CNS	central nervous system
COL1A1	alpha-1 type I collagen
COMT	catechol-O-methyltransferase
COX	cyclooxygenase
CPSQ	Comprehensive Pain and Symptoms Questionnaire
CREB1	cyclic AMP responsive element-binding protein 1
CRPS	complex regional pain syndrome
CSF	cerebral spinal fluid

CT	cycle threshold
δ	delta
DAG1	dystroglycan 1
DRG	dorsal root ganglion
DRPLA	dentatorubral-pallidoluysian atrophy
DUSP	deubiquitinating enzymes
ECM	extracellular matrix
EGFR	epidermal growth factor receptor
ERBB4	receptor tyrosine-protein kinase erbB-4
ERK	extracellular signal-regulation kinase
ETS1	protein C-ets-1
FDR	false detection rate
FM	fibromyalgia
FNDC1	fibronectin type III domain containing 1
GCH1	GTP cyclohydrolase 1
GnRH	gonadotropin-releasing hormone
GPCR	G protein-coupled receptors
G Protein	guanine nucleotide-binding protein
HC	healthy control
HLA	human lymphocyte antigen
HTR2A	serotonin receptor 2A
IASP	International Association for the Study of Pain
IBS	irritable bowel syndrome
ICHD-2	International Classification of Headache Disorders
ICI	ICI-118,511
i.c.v.	intracerebroventricular

IKK	I kappa B kinase
IL	interleukin
INS	insulin
INSR	insulin receptor
i.p.	intraperitoneal
IPD	idiopathic pain disorder
i.pl.	intraplantar
i.t.	intrathecal
ITGA5	integrin alpha 5
ITGB1	integrin beta 1
JAK-STAT	janus kinase and signal transducer and activator of transcription
Kegg	Kyoto Encyclopedia of Genes and Genomes
KRAS	GTPase KRas
LPAR1	lysophosphatidic acid receptor 1
LPS	lipopolysaccharide
MB-COMT	membrane bound catechol-O-methytransferase
MAP3K14	mitogen-activated protein 3-kinase 14
MAPK	mitogen-activated protein kinase
MAX	myc-associated factor X
MOA	monoamine oxidase
mRNA	messenger RNA
miRNA	microRNA
MPQ	McGill Pain Questionnaire
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NIH	National Institutes of Health
NK	natural killer

NK1R	neurokinin-1 receptor
NMDA	N-methyl-D-aspartate
NO	nitric oxide
NRS	numerical rating scale
NSAID	non-steroidal anti-inflammatory drug
OA	osteoarthritis
OPRM1	mu-opioid receptor
PAMP	pathogen-associated molecular patterns
PIK3CA	phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha
PILL	Pennebaker Index of Limbic Languidness
PLCB4	phospholipase C beta-4
pri-miRNAs	primary microRNAs
PRKCA	protein kinase C
PRKACB	cAMP-dependent protein kinase catalytic subunit beta
prop	propranolol
RAF1	RAF proto-oncogene serine/threonine-protein kinase
RIN	RNA integrity number
RISC	RNA-induced silencing complex
s.c.	subcutaneous
SCL-90R	Symptom Checklist 90-Revised
S-COMT	soluble catechol-O-methytransferase
SF12v2	Short Form 12 version 2
Shm	Sham
SLC6A4	solute carrier family 6 member 4
SNL	spinal nerve ligation
SNP	single nucleotide polymorphism

SR	SR59230A
SSRI	selective serotonin reuptake inhibitors
TGF-β	transforming growth factor beta
TMD	temporomandibular disorder
TMJ	temporomandibular joint
TNF	tumor necrosis factor
TRPV	transient receptor potential vanilloid cation channel V
VBD	vestibulodynia
Veh	vehicle

CHAPTER 1

INTRODUCTION

1.1 The Persistent Pain Epidemic

1.1.1 Definition and Impact of Persistent Pain

The International Association for the Study of Pain (IASP) describes pain as "an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage."[1] Pain exists prior to tissue damage as a warning sign, motivating an individual to withdraw from a stimulus that could cause further damage and encouraging the individual to avoid similar stimuli in the future. In the presence of tissue damage pain promotes protection of the injured region, allowing healing to occur.[1] Acute pain is therefore important and necessary for protection of the body.[1] In fact, Congenital Insensitivity to Pain (CIP) is a rare condition in which a person is unable to perceive pain and is therefore extremely susceptible to wounds and injuries, resulting in reduced life expectancy and increased susceptibility to disease.[2] In contrast, some individuals experience exaggerated pain in response to an acute stimulus or spontaneous pain in the absence of a stimulus. This type of pain, which is neither protective nor adaptive, is a hallmark of chronic pain conditions.

Chronic pain, commonly referred to as persistent pain, is defined by the IASP as pain that exists for an extended duration.[1] Persistent pain conditions represent one of the nation's most significant healthcare problems, affecting over 100 million Americans and costing the United States economy over $600 billion every year. Patients with persistent pain conditions experience uncontrollable and debilitating symptoms nearly six days of the week, often lasting for over five years. In fact, persistent pain affects more Americans than heart

disease, cancer, stroke and diabetes combined, accounting for 20% of medical visits and 10% of prescription drug sales.[3-5]

Persistent pain conditions may be categorized as neuropathic, inflammatory, or idiopathic. Neuropathic pain results from injury or disease that affects the peripheral, spinal, and/or supraspinal nervous systems. It is often accompanied by maladaptive changes to the somatosensory nervous system[1] such as alterations in action potential firing, synaptic transmission, synaptic connectivity and circuiting, and neuroimmune interactions. These alterations lead to amplification of pain. Examples of neuropathic pain include conditions such as chemotherapy-induced peripheral neuropathy, diabetic neuropathy, and spinal cord injury.[6] Animal studies utilize peripheral or spinal nerve ligations (SNLs) to mimic neuropathic pain conditions in humans.[7]

Inflammatory pain is characterized by an ongoing inflammatory response and nociceptive activation that may or may not exist as a result of tissue damage or injury. For example osteoarthritis is a persistent inflammatory condition that can develop as a result of trauma or infection to the joint, whereas rheumatoid arthritis is an autoimmune condition. Other examples of inflammatory pain conditions include complex regional pain syndrome (CRPS) and endometriosis. Animal studies utilize injections of inflammatory agents such as carrageenan, capsaicin, or complete Freund's adjuvant (CFA) to mimic inflammatory pain conditions.[7]

In contrast to neuropathic and inflammatory pain, idiopathic pain exists without underlying neural or tissue damage. Idiopathic pain is heterogeneous in nature and can be described as burning, itching, stinging, irritating, stabbing, and/or sharp. Pain severity and type can vary with factors such as age, gender, culture, and economic status.[8-10] Idiopathic pain disorders (IPDs) such as vestibulodynia (VBD), irritable bowel syndrome (IBS), temporomandibular disorder (TMD), and fibromyalgia (FM) are an example of idiopathic pain. Currently there are no existing animal models for idiopathic pain. Many models utilize

the aforementioned neuropathic and inflammatory animal pain models (*i.e.,* SNLs, carrageenan injections) to study IPDs. These do not adequately represent the absence of neural and/or tissue damage that defines IPDs. This topic is discussed in greater detail in Section 1.3.3.

1.1.2 Characteristics of Idiopathic Pain Disorders

While clinical manifestations of idiopathic pain are heterogeneous, IPDs are characterized by a state of pain amplification, psychological distress, and enhanced inflammation:

i. Pain Amplification

As previously mentioned, IPDs are characterized by pain amplification that occurs in the absence of an apparent cause.[11] The pain may be continuous or intermittent and it may be provoked, only present with an accompanying stimulus (*i.e.,* sexual intercourse or tampon insertion in VBD) or spontaneous, occurring in the absence of a stimulus.[12] Pain may be localized to one area of the body in IPDs such as TMD, VBD, IBS, low back pain, and migraines; or it can be distributed across several regions of the body in IPDs such as FM. Persistent idiopathic pain at one site often co-occurs with pain in one or more distinct regions of the body.[13] Additionally, IPD patients often report enhanced widespread bodily pain as well as decreased thresholds in response to stimuli at various sites. For example, TMD patients are more likely to report a history of headaches or back pain, and often possess a greater number of irritable bowel syndrome symptoms.[14] Patients with migraines report skin hypersensitivity as well as decreased pain thresholds at various sites of the body.[15]

ii. Psychological Mood

Patients with IPDs also frequently suffer from severe psychological and socioeconomic consequences in addition to their physiological symptoms.[16,17] Depression is one of the many psychiatric comorbidities that is observed alongside idiopathic pain, and it

has been linked to back pain,[18] migraines,[19] FM,[20] VBD[21] and IBS.[22] Uncontrolled pain at severe levels, particularly when in coexistence with depression, increases the risk of suicide.[23] Anxiety and anhedonia are also among psychological conditions that commonly coexist with IPDs.[24,25] Physical and emotional pain are thought to share neural pathways and connectivity, which could explain why psychological distress frequently co-occurs with IPDs. For example, exposing a subject to noxious heat and unpleasant pictures causes overlapping activation of the brain.[26] In line with this, psychological conditions such as depression are known to intensify pain sensitivity,[27] while positive emotions can help to decrease pain perception.[28] The relationship between a psychological condition such as depression and physical pain is bidirectional. Each can intensify the other, leading to a vicious "mutual maintenance" cycle that promotes distress and disability in IPD patients.[25,29] This mutually reinforcing relationship makes treatment an even greater challenge for clinicians.

iii. Immune Response

IPDs are also associated with abnormalities in immune signaling. In a healthy individual an interactive network of organs, factors, and cytokines, which comprise the immune system, work to defend the body from pathogens and disease. Cells that serve the immune system include mononuclear cells (macrophages, monocytes, T cells, natural killer (NK) cells, leukocytes) and granulocytes (neutrophils, eosinophils, basophils). Immune cells secrete cytokines, which are small messenger proteins that can alter the behavior of surrounding cells by influencing processes such as cell activation, division, or movement.[30] Following injury, immune cells are activated within minutes.

In a healthy individual, pro-inflammatory cytokines such as monocyte chemotactic protein (MCP-1), interleukin-1β (IL-1β), IL-6, and tumor necrosis factor α (TNFα) and anti-inflammatory cytokines such as IL-1 receptor antagonist (IL-1ra or IL-RN), IL-4, and IL-10 work together in a concerted balance to regulate inflammatory processes following tissue

damage. In patients with IPDs, cytokine expression is altered.[31] For example, TMD is associated with elevated levels of MCP-1 and IL1-ra,[32] FM with elevated levels of IL-6 and IL-8,[33] and VBD with elevated levels of TNFα. A reduction in anti-inflammatory cytokine levels has similarly been associated with IPDs, particularly when the pain is widespread or exists at multiple sites.[32,33] Collectively, these studies suggest that immune cell dysregulation, and/or an imbalance between pro- and anti- inflammatory cytokines, may facilitate the development of IPDs.[34] Cytokine accumulation following injury may lead to IPDs *via* peripheral and/or central sensitization.[35]

Peripheral sensitization begins when inflammatory mediators such as cytokines interact with primary afferent neurons in the tissue. Primary afferent Aβ fibers are responsible for transmitting non-noxious stimuli, whereas Aδ and C fibers transmit noxious signals. Aδ fibers are lightly myelinated with a small diameter, responsible for sharp pain immediately following injury. C fibers are unmyelinated with a very small diameter, responsible for slow, burning pain. Aδ and C fibers synapse with second-order afferent neurons in the dorsal horn of the spinal cord, relaying sensory information to supraspinal structures including the thalamus and the brainstem.[36] Following exposure to inflammatory mediators and/or damaged tissue, nociceptor sensitivity is enhanced. Activation thresholds are reduced, resulting in amplified nociceptor responsiveness.[36,37] Many pathways, such as the mitogen-activated protein kinase (MAPK)[38] and cyclic adenosine monophosphate kinase (cAMP-PKA)[39] pathways are expected to play a role in the sensitization downstream from enhanced pro-inflammatory cytokine production. In an healthy individual, peripheral sensitization is usually coupled to the presence of an acute peripheral noxious stimuli.[40] Peripheral sensitization that continues to exist in the absence of a stimulus, however, is pathological and is associated with IPD onset and idiopathic pain localized to the area of sensitization.[41,42]

Sustained or repetitive activation of primary afferents can also alter central pathways, leading to central sensitization. In central sensitization, neurotrophic factors such as substance P and brain-derived neurotrophic factor (BDNF) induce long-lasting spinal excitability. Further, wide diameter Aβ fibers, which normally transmit non-noxious signals, begin to act like C fibers. As a result, normally innocuous stimuli such as light touch or pressure becomes painful.[36,37,40] Central sensitization is a normal response that can be induced in healthy individuals *via* mechanical, thermal, or chemical activation of nociceptors. This sensation generally lasts for many hours beyond the inducing stimulus. Unsurprisingly, central sensitization can also become pathological, if it exists for an extended or persistent duration.[42] Central sensitization may explain why patients with one IPD are susceptible to developing IPDs at separate, distinct areas. Further, it may explain why patients with IPDs such as TMD,[43] FM,[44] neck pain,[45] and headache[46] demonstrate widespread mechanical and/or thermal pain sensitivity. To summarize, IPDs are associated with immunological abnormalities and pathological peripheral and central sensitization.

1.1.3 Vestibulodynia Represents a Common, yet Understudied Idiopathic Pain Disorder

VBD is an understudied IPD characterized by debilitating pain or discomfort to the vulvar region and is a main focus of the studies outlined in Chapter 4 of this dissertation. Like other pain conditions, VBD exists in the absence of a visible cause or inciting neurological disorder.[47] The vulvar pain experienced by patients with VBD is often described as burning, stinging, irritating, or raw[48] and is associated with substantial disability.[49] The pain may be provoked by sexual intercourse or activities such as walking, or it may be completely unprovoked and continuous in the absence of a stimulus.[50] Like other IPDs, VBD can have a significant effect on a woman's psychological health. In addition to the common psychological comorbidities outlined in section 1.1.2, women with VBD commonly suffer

from problems with body image, sexual function, and social relationships, leading to a significant decrease in their quality of life.[49-51]

Despite the physical and emotional characteristics of VBD, patients frequently fail to seek treatment for fear of neglect or skepticism.[52] Many professionals in the medical community believe that VBD is solely a psychological problem, and others misdiagnose and/or mistreat patients as a result of not understanding of the condition.[49] Though studies by the National Institutes of Health (NIH) report that over 15% of American women seek treatment for VBD, population-based studies predict that close to 40% of women actually suffer from the condition.[52] In this sense research and care for VBD is far behind that for conditions such as TMD and FM. A greater awareness and understanding of VBD is necessary not only to improve treatment for this condition, but also to encourage individuals suffering from vulvar pain to seek care for their symptoms.[53]

1.1.4 Current Treatment Strategies for VBD and Related Idiopathic Pain Disorders

Current pharmacological treatments for VBD and other IPDs are rarely effective and often associated with adverse side effects that can be severe and at times life threatening. Opioids, one of the most commonly prescribed drugs for idiopathic pain, reduce pain by activating opioid receptors, thus mimicking the actions of endorphins.[54] Unfortunately opioids are associated with several adverse side effects including dependence, addiction, gastrointestinal problems, altered mental state, muscle rigidity, nausea and respiratory depression.[55-58] Furthermore, long-term use of opioids is associated with increased pain sensitivity and may actually enhance pain further through a phenomenon known as opioid-induced hyperalgesia.[59,60]

Non-steroidal anti-inflammatory drugs (NSAIDs), also commonly prescribed for patients with IPDs such as VBD, reduce pain by inhibiting cyclooxygenases (COX-1 and COX-2) and subsequently preventing the synthesis of pro-inflammatory prostaglandins. NSAID use has been associated with gastrointestinal ulcers, renal dysfunction,

cardiovascular risk, impaired hemostasis and asthma.[61] Many of the other drugs used for the treatment of idiopathic pain, such as tricyclic antidepressants, selective serotonin reuptake inhibitors (SSRIs), antiepileptic drugs, and cannabinoids, are mitigated by similar side effects and marginal efficacy.[61] Topical applications of substances or drugs such as estrogen, lidocaine jelly, nitroglycerine, amitriptyline, baclofen, or capsaicin or injections of triamcinolone or botulinum toxin A may help to reduce pain in patients, but the relief is often short-lived.[50] Current research is focused on the development of drugs as well as non-pharmacological treatment strategies to overcome these limitations.

Non-pharmacological interventions for IPDs can be behavioral, cognitive, integrative or physical in nature.[62] Unlike drugs, which target the biological causes of pain, these interventions work to address the psychological and social consequences of idiopathic pain.[63] Examples include behavioral therapy, cognitive-behavioral therapy, hypnosis, guided imagery, diaphragmatic breathing, muscle relaxation, mindfulness-based stress reduction, acupuncture, stretching, exercising, and physical therapy.[62] VBD-specific examples include physical therapy that targets the pelvic floor, pelvic floor surface electromyography and biofeedback, a low-oxalate diet with calcium citrate supplementation, surgery (*e.g.,* localized excision, vestibulectomy, perineoplasty), and sexual counseling.[50] Though non-pharmacological interventions for IPDs usually are not sufficiently effective alone, they can be used in combination with pharmacologic treatment strategies to enhance drug efficacy.[62] Still, future research is necessary to develop better therapeutics as well as to understand individual variability that may impact the efficacy of treatment for VBD and other IPDs.

1.2 Known Causes and Correlates

1.2.1 Environmental Factors

Alterations in pain, mood, and inflammation that are observed in patients with IPDs are likely due to both environmental and genetic factors. In some cases IPDs are thought to

develop as a result of a previous trauma, injury, or event. For example, 30-50% of patients suffer from idiopathic pain between one and one half and two years after a surgery such as hernia repair, amputation, mastectomy, or thoracotomy.[64-67] The presence of severe acute pain in post-operative patients 24 hours following surgery, both at rest and following movement, can be predictive of IPDs later in life.[65] Infections have also been associated with IPDs.[68,69] Many VBD patients have a history of frequent yeast infections.[50,70] Other environmental stressors that commonly lead to idiopathic pain or IPDs include car accidents,[71,72] bone fractures,[73,74] physical abuse,[75] sexual abuse,[76,77] and cancer.[78-80] Scientists hypothesize that traumatic events or environmental factors such as these may result in inflammation or nerve damage that never resolves.[81] Chapter 1.1.2 provides a detailed description of possible mechanisms that may facilitate idiopathic pain following traumatic events or injuries. Collectively, these studies provide strong evidence for a relationship between environmental stressors and idiopathic pain. Despite this evidence, the development of an IPD cannot be attributed to environmental factors alone.

1.2.2 Genetic Factors

Not all individuals develop one or more debilitating IPDs following an event such as surgery or a car accident. This may be explained by genetics which, in conjunction with the environmental factors listed above, can also play a vital role in the development of idiopathic pain. Hundreds of pain- and analgesia-relevant genes have been identified from gene microarray studies. Acute, inflammatory, cancer, musculoskeletal, neuropathic, idiopathic, visceral, widespread, and experimental pain phenotypes have been associated with various genes. Mouse studies predict that the heritability for nociceptive and analgesic sensitivity is somewhere between 28-76%.[82,83]

The most popular candidate genes for pain include those that code for catechol-O-methyltransferase (COMT), GTP cyclohydrolase 1 (GCH1), human lymphocyte antigen (HLA), serotonin receptor 2A (HTR2A), IL-1A, IL-1B, IL-1RA, μ opioid receptor (OPRM1),

solute carrier family 6 member 4 (SLC6A4), transient receptor potential cation channel V1 (TRPV1), and TNF. Unsurprisingly, many of these genes are involved in the activation or expression of receptors (*e.g.,* OPRM1, TRPV1), molecules (*e.g.,* ILs, TNF) or pathways involved in pain processing.[81] *COMT* is one of the most widely studied genes in the context of pain. *COMT* is of particular interest to the studies outlined in this dissertation and is discussed further in Chapter 1.3. Of note, genetic associations have also been established for several psychological traits and conditions that can influence pain perception and the susceptibility of developing IPDs (See Chapter 1.1.2 for more information on the psychological traits and conditions associated with IPDs). For example the heritability of depression is estimated to be within 50-70%. Genes that contribute to pain perception and/or a pained psychological state may influence an individual's susceptibiltiy of developing persistent idiopathic pain and IPDs.

1.2.3 Epigenetic Factors

Environmental stressors or factors may be involved in the activation of idiopathic pain or onset of IPDs in individuals who have genetic predispositions. Perhaps the best evidence of this comes from studies that demonstrate significant differences in inflammatory and persistent pain phenotypes in monozygotic twins.[84,85] Though twins may have identical or similar epigenomes at birth, they are later subjected to environmental differences and, consequentially, epigenetic differences that may or may not activate their genetic predisposition to develop idiopathic pain.[86]

The field of epigenetics explores the interactions between genes and the environment. Emerging evidence suggests that epigenetic mechanisms can silence expression of genes that are pro- or anti-nociceptive, thus altering pain pathways.[86,87] Factors such as those listed in Chapter 1.2.1 as well as environmental toxins, medications, diet, psychological stressors, age, nutrition, and social context can alter epigenetic processes, including DNA methylation, histone acetylation, and RNA interference.[74,88-90]

Epigenetic modifications like these likely play a pivotal role in the development of IPDs, as they can impact pro- and anti-inflammatory cytokine expression, steroid responsiveness, and opioid sensitivity.[86] To summarize, IPDs are a product of genetic and environmental factors, an interaction that is important to consider in future studies (Figure 1.1).[91]

i. MicroRNAs

MicroRNAs (miRNAs) are a type of epigenetic regulation that may affect pain-relevant processes.[67] MiRNAs are small, non-coding pieces of RNA that are usually nineteen to twenty-five nucleotides in length and can regulate gene expression by binding to downstream mRNA targets.[92] Primary miRNAs (pri-miRNAs) are initially transcribed in the nucleus, then processed into precursor pre-miRNAs by the "Drosha" endonuclease. Intracellular transport protein exportin 5 facilitates the export of pre-miRNAs into the cytoplasm, where they are converted into mature miRNAs by the endonuclease "Dicer". Double-stranded, mature miRNAs become single-stranded following attachment to the RNA-induced silencing complex (RISC). At this point miRNAs can regulate gene expression, either by completely binding to their downstream mRNA targets and thereby cleaving the transcript or by incompletely binding and thereby inducing translational repression.[92,93] MiRNAs represent a complex regulatory network, as each individual miRNA binds to several downstream mRNAs and, similarly, each mRNA transcript complements several miRNAs.[92]

Dysregulation of miRNAs can cause subsequent dysregulation of downstream pathways and processes, often resulting in disease. MiRNA dysregulation has been observed in various conditions including multiple sclerosis,[94] peripheral artery disease,[95] major depressive disorder,[96] cardiovascular disease,[97] liver injury,[98] and cancer.[99] MiRNAs may represent a therapeutic target for many of these conditions. In fact, phase two clinical trials are currently assessing the efficacy of a miRNA-based therapeutic for the treatment of cancer. The aim of this therapeutic, known as "miRNA replacement therapy," is to re-introduce miRNAS into diseased cells where they are downregulated.[100] MiRNAs may also

be used in the future to diagnose disease. Circulating miRNAs are stable in serum and plasma, making them ideal biomarkers.[101]

ii. MicroRNAs Associated with Pain and Inflammation

Dysregulation of miRNAs that are involved in pain- and inflammation-relevant processes may facilitate the development of persistent idiopathic pain.[93] Several miRNAs are known to play a role in the development and normal function of the immune system. Abnormal immune system function can lead to overproduction of inflammatory mediators (*i.e.,* cytokines), contributing to IPDs.[102,103] Many studies have confirmed the importance of miRNAs in regulating immune cell development and fine-tuning the immune cell response.

Granulocytes (neutrophils, eosinophils, basophils) and mononuclear cells (monocytes, lymphocytes, macrophages) each play important roles in defending the body (see Chapter 1.2.2). MiR-223 plays an important role in granulocyte differentiating, as well as in the development and function of neutrophils, which act as the first line of defense against pathogens. Other miRNAs, including miR-17-5p, miR-20a, miR-106a, and miR-424 are involved in the regulation and differentiation of monocytopoiesis, or the production of mononuclear cells. Several different miRNAs are either upregulated or downregulated in response to pathogen-associated molecular patterns (PAMPs) such as lipopolysaccharide (LPS) stimulation in mononuclear cells. Activation of toll-like receptors (TLR2, TLR4, TLR5) or inflammatory cytokines (IL-1 β) affects the expression of miR-146a and miR-147 in mononuclear cells. MiR-21 and miR-9 are each responsible for the negative regulation of LPS-activated inflammatory signaling. MiR-155 and miR-17-92 play distinct roles in lymphocyte development and function.[104] Let-7i and miR-125b are decreased during innate immune responses. A summary of miRNA regulation of immune cells can be found in Figure 1.3A.

MiRNAs have also been directly linked to the expression of inflammatory cytokines. Many miRNAs, including miR-29a,[105] miR-146a,[106] miR-124,[107] and miR-93[108] are involved in

the regulation of inflammatory cytokine secretion. Additionally, miRNAs are regulators of nuclear factor kappa-light-chain-enhancer of B cells (NF-κB), which controls inflammatory genes and cytokine secretion and is chronically active in many inflammatory diseases.[109,110] Collectively this demonstrates that miRNAs play an important role in the immune system and can alter the immune response.

Aberrant miRNA profiles have been observed in various rodent models of pain and inflammation. This includes inflammatory pain models such as CFA,[111,112] carrageenan,[113] and capsaicin[112] injections as well as neuropathic pain models such as peripheral nerve ligations and SNLs.[112,114,115] Rodent studies have also linked miRNA expression to nociceptor excitability and pain thresholds.[116] Furthermore, intrathecal injections of miR-103 and miR-124, which were previously identified as regulators of pain signaling, have been shown to prevent and alleviate pain in rodent models.[117,118] These are the first studies to support the use of miRNAs as treatment for pain.[119]

MiRNA dysregulation has similarly been associated with pain-related diseases in humans. For example, miR-146a is significantly upregulated in peripheral knee joint tissues of osteoarthritis (OA) patients. It regulates pain-related factors, modulates inflammatory molecules, and may be used in the future as a treatment target for OA.[120] Likewise, miRNA dysregulation has been linked to rheumatoid arthritis[104] and complex regional pain syndrome (CRPS). In CRPS, miRNA profiles can be used to stratify patients into sub-groups that may respond to treatment plans differently.[121] More recently, miRNA dysregulation has been linked to IPDs such as fibromyalgia,[122] bladder pain syndrome (BPS),[123] and migraines.[124] A list of additional pain-relevant animal models and clinical conditions that have been linked to miRNA dysregulation is shown in Figure 1.3B.

To summarize, miRNA dysregulation plays an important role in many pain-relevant processes and conditions. The dysregulation of pain-regulating miRNAs may facilitate the development of idiopathic pain.[93] Additional research is required to understand the

relationship between miRNAs and pain and how that relationship might be exploited to develop more effective therapeutic strategies for IPDs. The studies presented in this dissertation aim to elucidate the role of miRNAs in idiopathic pain, utilizing experiments in an animal model as well as in clinical IPDs.

1.3 The Contribution of Catechol-O-methyltransferase

1.3.1 Catechol-O-methyltransferase and Persistent Pain

As discussed in Chapter 1.2.1, there is strong evidence of a role for COMT in persistent and idiopathic pain. COMT is an enzyme that metabolizes catecholamines such as dopamine, epinephrine, and norepinephrine. Its active site consists of an S-adenosyl-L-methionine (AdoMet) binding site and a catalytic site. In the presence of Mg^{2+} COMT catalyzes the transfer of the AdoMet methyl group to one of the two hydroxyl groups of the catechol. The COMT gene codes for two forms: membrane bound (MB-COMT) and soluble (S-COMT). S-COMT is dominantly expressed in the majority of tissues; however, in the brain 70% of total COMT is MB-COMT.[125] COMT expression varies across tissue types as a result of tissue-specific transcription factor-mediated gene regulation.[126]

Decreased activity of COMT has been directly linked to pain-relevant conditions such as facial pain.[127,128] Diminished COMT activity leads to abnormalities in catecholamine levels and physiology,[125] which have also been linked to pain and IPDs. In fact, previous studies have utilized catecholamine excretion as a measure of pain-relevant conditions such as rheumatoid arthritis.[129] Patients with FM demonstrate increased levels of norepinephrine, which increase further in response to enhanced pro-inflammatory cytokine expression.[130,131] Patients with myofacial pain[132,133] and chronic bladder pain[134] similarly demonstrate sustained elevation of catecholamines and augmented sympathetic signaling. Catecholamine abnormalities have also been linked to psychological characteristics associated with IPDs, such as stress,[129,135] anxiety,[136,137] and depression.[138]

1.3.2 Catechol-O-methyltransferase is Influenced by Genetic and Environmental Factors

Historically, COMT research has focused on a common single nucleotide polymorphism (SNP) in the gene locus (rs4680), which causes a valine (Val) to methionine (Met) substitution at codon 158 and leads to a four-fold reduction of the enzyme. Individuals with the Met/Met genotype have reported higher sensory and affective pain ratings and have a higher regional density of μ-opioid receptors. Additionally, this polymorphism can influence the efficacy of morphine treatment for pain.[139] This polymorphism alone, however, has not been able to fully account for individual variation in pain sensitivity, as results across studies have been largely inconsistent.[140]

Our laboratory has also demonstrated the importance of haplotypes consisting of rs4680 along with three other common SNPs (rs6269, rs4633, rs4818, and rs4680) to persistent pain. We identified three haplotypes in the COMT gene locus, which account for variation in pain sensitivity among individuals: the G_C_G_G haplotype, associated with a low pain sensitivity (LPS) phenotype; the A_T_C_A haplotype, associated with an average pain sensitivity (APS) phenotype; and the A_C_C_G haplotype, associated with a high pain sensitivity (HPS) phenotype. The LPS haplotype provides 4.8 times higher levels of COMT activity compared with the APS haplotype. The HPS haplotype provides 11.4 times lower levels of COMT activity compared with the LPS haplotype. In addition to increased experimental pain sensitivity, the HPS phenotype is associated with TMD onset. During a three-year observational period, individuals with only HPS and/or APS haplotypes were more than twice as likely to develop TMD than those with at least one LPS haplotype.[141] Individuals with HPS COMT haplotypes are also more likely to develop pain or IPDs following traumatic events such as surgeries,[142,143] car accidents,[144] and orthodontic treatment.[90] Additionally, COMT haplotypes are associated with variance in pain severity for patients with FM[145,146] as well as postsurgical shoulder pain.[143]

Variability in the COMT gene also determines efficacy in the treatment of pain disorders by propranolol, a non-selective beta-adrenergic receptor (βAR) antagonist that effectively blocks COMT-dependent mechanical and thermal pain in rodents[147] and is commonly used to treat chronic daily headaches in the clinic.[148] Propranolol substantially improves experimental and clinical pain in TMD subjects not carrying a LPS haplotype. The pain is however only moderately improved in LPS heterozygotes, and not affected at all in LPS homozygotes.[140]

To summarize, decreased COMT activity levels lead to increased catecholamine levels, resulting in heightened pain sensitivity (Figure 1.2). Genetic variability in the COMT gene signficantly alters an individual's response to experimental pain, susceptibility to IPDs and responsiveness to treatment. This is an important consideration that must be considered by clinicians who treat individuals with idiopathic pain. Future research should focus on elucidating the mechanisms underlying COMT-dependent pain.

1.3.3 An Animal Model of COMT-Dependent Pain

Though animal models have been instrumental in developing a better understanding of the mechanisms underlying pain, many existing models do not accurately reflect human IPDs. Instead, they often utilize inflammatory agents to provoke pain. Many of these models require persistent injections or applications of the chosen drug or chemical. Other animal models induce pain by inflicting nerve damage. These models, though useful for the study of inflammatory or neuropathic pain, do not accurately represent IPDs (Chapter 1.1.1). As a result, many potential analgesics have failed in the clinic despite strong efficacy in a pre-clinical animal model.[7] Our laboratory has developed a rat model of persistent idiopathic pain in which a COMT inhibitor is administered to rats to mimic the endogenously low levels of COMT activity observed in patients (Chapters 1.3.1 and 1.3.2 explore the role of COMT in persistent idiopathic pain.) We believe this model represents a clinically-relevant form of pain that more closely mimics the experience of humans with IPDs than is the case for other

animal models that rely on repeated administration of noxious stimuli. We have utilized this model to gain a better understanding of the mechanisms underlying COMT-dependent pain.

Consistent with clinical IPDs, administration of a COMT-inhibitor to rats results in mechanical and thermal pain and alters cognitive-affective behaviors linked to pain (*e.g.,* avoidance of painful heat and bright light).[147,149,150] Because catecholamines stimulate the sympathetic nervous system *via* activation of adrenergic receptors (ARs), pharmacologic studies were utilized to assess the efficacy of alpha (α_1 and α_2) and beta (β_1, β_2, and β_3) adrenergic as well as dopaminergic receptor antagonists in blocking OR486-induced pain. Results demonstrated that OR486-induced pain is blocked by the non-selective β-adrenergic receptor (βAR) antagonist propranolol or by the combined administration of selective β_2- and β_3AR antagonists. In contrast OR486-induced pain is not blocked by β_1-adrenergic, α-adrenergic, or dopaminergic receptor antagonists.[147] These results are in line with those from clinical studies, showing that propranolol alleviates pain among FM and TMD patients.[140,151] Collectively these studies suggest that increased catecholamine levels, resulting from reduced COMT activity, drive pain *via* β_2- and β_3ARs.

i. β-Adrenergic Receptors and COMT-Dependent Pain

ARs are G protein-coupled receptors (GPCRs), responsible for transmitting signals from extracellular ligands to the intracellular environment to control various cellular events and physiological processes. Though several ARs exist—alpha-adrenergic (α_1 and α_2), beta-adrenergic (β_1, β_2, and β_3) and dopaminergic—only β_2- and β_3ARs are known to contribute to COMT-dependent pain (see Chapter 1.3.3). βARs were first discovered in 1948 and have been heavily studied in the context of heart disease. Though βAR antagonists, otherwise known as β-blockers, were developed for the treatment of heart failure in 1975,[152] it was not long before their efficacy was discovered for pain reduction in conditions such as arthritis and joint pain.[153-155] Today they represent a promising possibility for the treatment of IPDs such as TMD and FM.[140,151]

Like all GPCRs, βARs have seven transmembrane-spanning α-helices, which fold to create three extracellular and three intracellular loops. Upon activation β_2- and β_3ARs couple to the G_s guanine nucleotide-binding protein (G protein), activating adenylyl cyclase and thereby leading to the formation of cAMP and activation of PKA. This represents a specific and rapid signaling cascade.[156,157] G_s interaction sites for both β_2- and β_3ARs are located in the membrane proximal regions of the second and third intracellular loops as well as at the carboxy-terminal domains.[157] Although many of the actions of both β_2- and β_3ARs are mediated by G_s proteins, studies have demonstrated that both receptor types can also couple to G_i proteins, leading to the activation of extracellular signal-regulation kinase (ERK) and MAPK pathways.[156,158]

β_2- and β_3ARs are expressed in peripheral and central regions where they could drive pain. β_2ARs are located on peripheral terminals[159-163] and cell bodies[164-166] of primary afferent nociceptors, keratinocytes,[167-169] immune cells,[170-173] and adipocytes[174] in the periphery and neurons[175,176] and glial cells[177] in the central nervous system. β_3ARs are located on primary afferent nociceptors,[178] adipocytes,[174] and immune cells[171,172] in the periphery and noradrenergic neurons in the brain.[179] A site of action for the β_2- and β_3ARs that mediate COMT-dependent pain has yet to be determined. The studies presented in this dissertation aim to identify a site of action for the βARs receptors that mediate COMT-dependent pain using clinically-relevant rodent models of COMT-dependent pain that improve upon those used in prior studies.

ii. MicroRNAs and COMT-Dependent Pain

It is important to note that miRNAs, which are discussed in detail in Chapter 1.2.3, may also play a role in COMT-dependent pain. It is likely that COMT-dependent pain is associated with the dysregulation not only of miRNAs involved in pain and immune processes, but also of those responsible for the regulation of catecholaminergic and/or βAR signaling. For example, previous studies have demonstrated a role for the miRNA let-7 in

regulating β_2AR expression.[180] Furthermore, βAR stimulation induces expression of miR-21[181] as well as miR-199a-5p.[182] It is thought that the cardioprotective effects of βAR antagonist propranolol are in part a result of miR-1 downregulation.[183] Several miRNAs have been associated with reduced catecholamine sensitivity in the heart, a characteristic of chronic heart failure.[184] Though many ongoing studies are examining the relationship between miRNAs and βAR-mediated cardiac disease; the relationship between miRNAs, βAR signaling, and COMT-dependent pain has yet to be studied.

The studies presented in this dissertation aim to elucidate the role of miRNAs not only in persistent idiopathic pain, but also specifically in COMT-dependent pain.

1.4 Summary and Specific Aims

Though IPDs are heterogeneous in nature, they are generally characterized by a physical state of pain amplification, psychological distress, and enhanced inflammation (Chapter 1.1.2). Persistent idiopathic pain manifests without an apparent cause, but it is facilitated by both environmental (Chapter 1.2.1) and genetic (Chapter 1.2.2) factors. One gene of particular interest codes for COMT and has been linked to increased pain sensitivity and susceptibility to developing IPDs (Chapter 1.3). Though studies have demonstrated that COMT-dependent pain is mediated *via* β_2- and β_3ARs, the site of action whereby these receptors mediate COMT-dependent pain remains unknown (Chapter 1.3.3). Chapters 2 and 3 therefore aimed to determine the site of action whereby β-adrenergic systems drive persistent COMT-dependent pain. We hypothesized that peripheral, spinal, and supraspinal βARs would contribute to COMT-dependent pain. We found that peripheral but not spinal or supraspinal β_2- and β_3ARs contribute to the initiation, but not the maintenance, of COMT-dependent pain.

Epigenetic modifications such as miRNA regulation can be initiated by environmental factors and may also facilitate the development of idiopathic pain, particularly in genetically

susceptible individuals (Chapters 1.2.3 and 1.3.3). Though emerging evidence suggests a role for miRNAs in idiopathic pain, the relationship between miRNAs and COMT-dependent pain has not been widely studied. Chapter 4 aimed to identify miRNAs associated with IPDs. We hypothesized that miRNA dysregulation would be associated with idiopathic pain in a clinical setting. As hypothesized, we observed miRNA dysregulation in patients with IPDs. We also found that miRNAs may be useful for distinguishing between separate subtypes of IPDs. Collectively, the present studies may help to inform future care for patients by elucidating the etiologies and identifying targets for treatment of idiopathic pain.

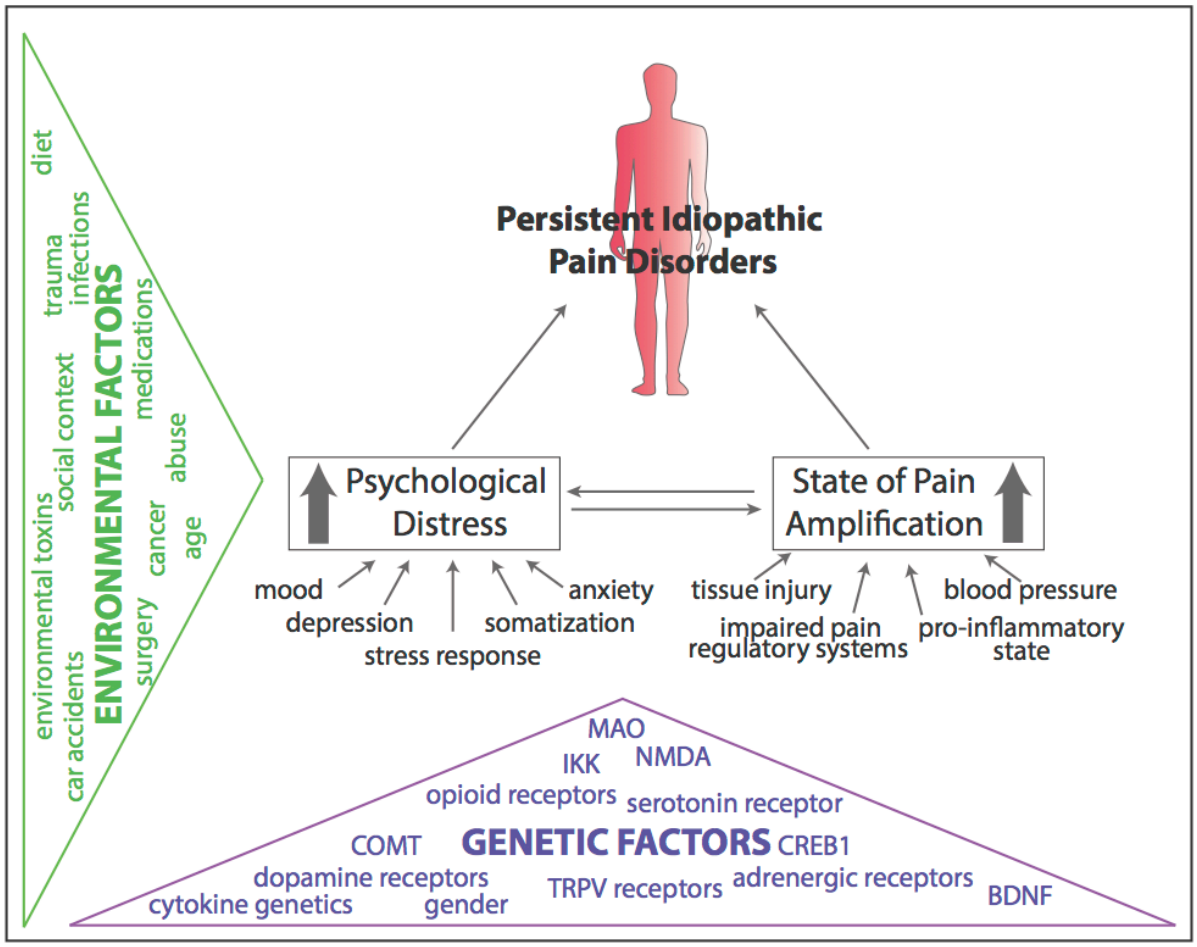

Figure 1.1 Idiopathic pain disorders are influenced by environmental and genetic factors. This model depicts environmental and genetic factors that determine an individual's psychological profile and pain amplification status. These two domains can influence the risk of onset and maintenance of IPDs in patients. Adapted from: "Idiopathic pain disorders: Pathways of vulnerability" by L.L. Diatchenko, 2006, *PAIN*.[91] Abbreviations: brain-derived neurotrophic factor (BDNF), catechol-O-methyltransferase (COMT), cyclic AMP responsive element-binding protein 1 (CREB1), idiopathic pain disorder (IPD), I kappa B kinase (IKK), monoamine oxidase (MAO), N-methyl-D-aspartate (NMDA), transient receptor potential vanilloid (TRPV).

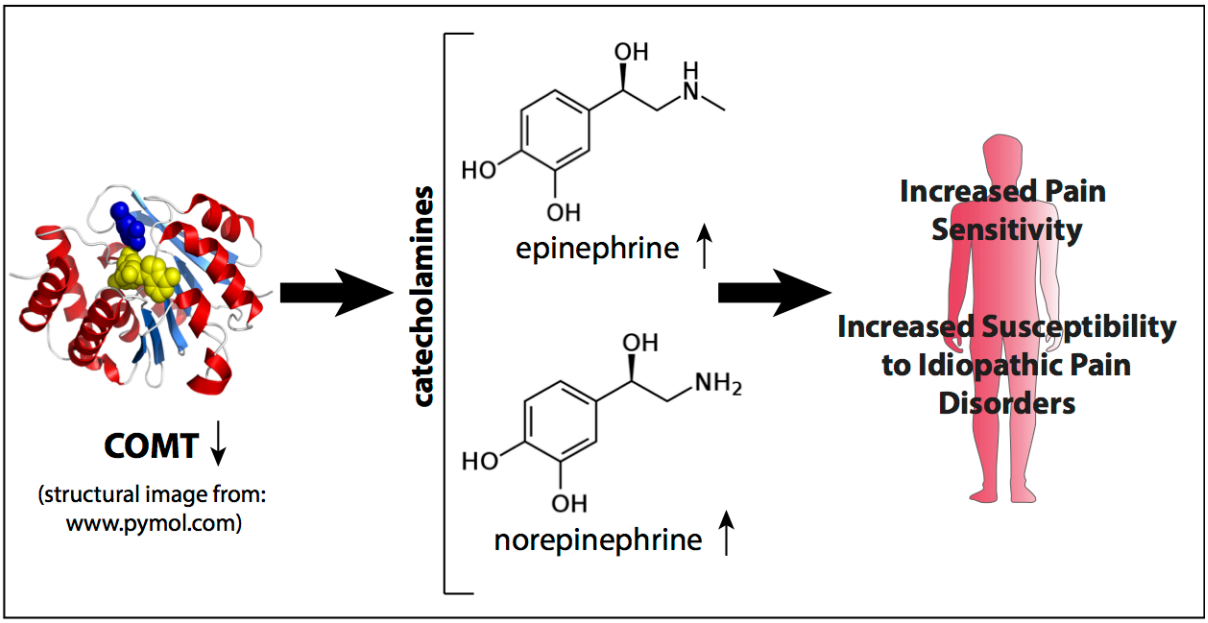

Figure 1.2 Decreased COMT activity is associated with idiopathic pain disorders. Genetic variability in the COMT gene can result in decreased levels of COMT activity and subsequently increased levels of circulating catecholamines (*e.g.,* epinephrine, norepinephrine), contributing to the development of IPDs. Abbreviations**:** catechol-O-methyltransferase (COMT), idiopathic pain disorders (IPDs).

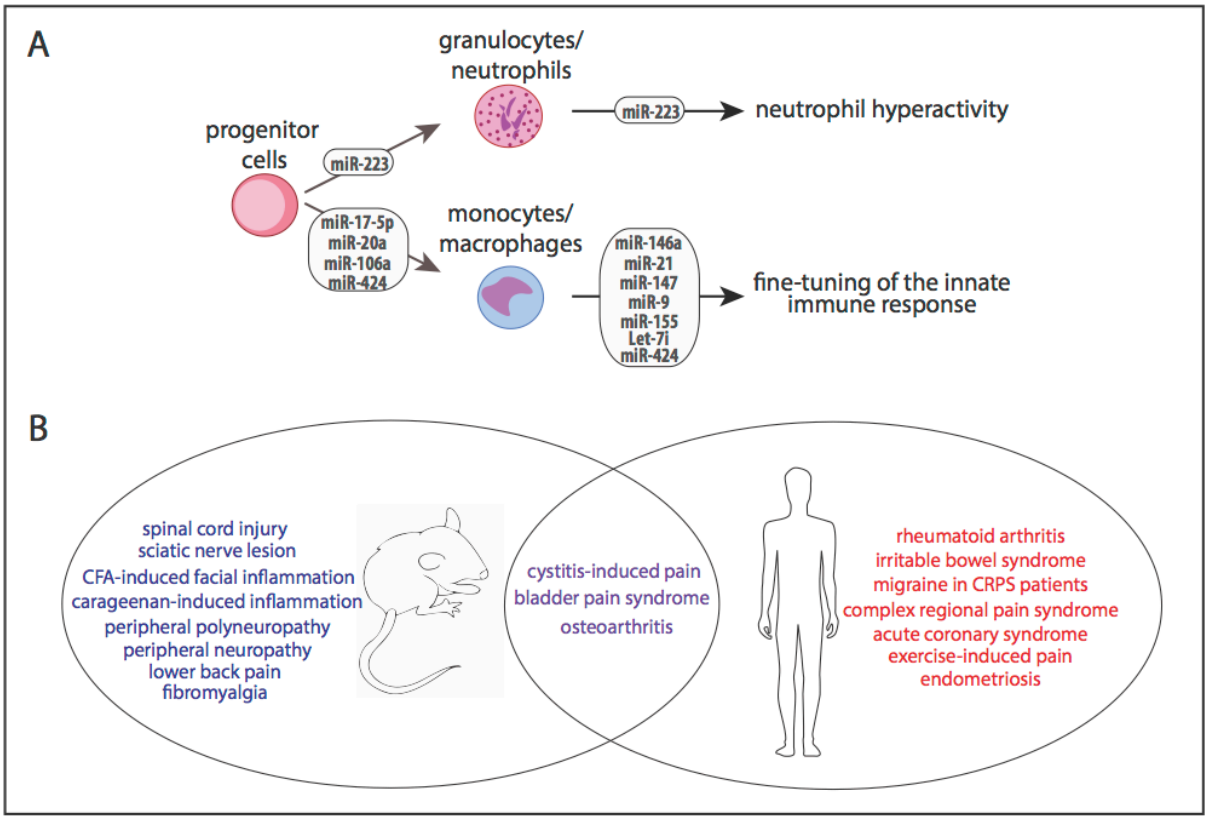

Figure 1.3 MicroRNA dysregulation can affect immune cell function and is associated with pain-relevant animal models and clinical conditions. (A) MiRNAs play an important role in the development and function of immune cells, subsequently fine-tuning the innate immune response and protecting the host from injury. Dysregulation of the miRNAs that are involved with immune system regulation may result in chronic inflammation and pain. Adapted from: "MicroRNA, a new paradigm for understanding immunoregulation, inflammation, and autoimmune diseases" by R. Dai and S.A. Ahmed, 2011, *Translational Research*.[104] **(B)** MiRNA dysregulation has been associated with various animal models of pain as well as clinical pain-relevant conditions. Blue = animal models of pain, red = clinical pain conditions, purple = animal models of pain in addition to clinical pain conditions. Adapted from: "MicroRNAs as modulators and biomarkers of inflammatory and neuropathic pain" by H.H. Andersen, M. Duroux, and P. Gazerani, 2014, *Neurobiology of Disease.* Abbreviations: complete Freund's adjuvant (CFA), complex regional pain syndrome (CRPS), microRNA (miR).

CHAPTER 2

PERSISTENT CATECHOL-O-METHYLTRANSFERASE-DEPENDENT PAIN IS INITIATED BY PERIPHERAL BETA-ADRENERGIC RECEPTORS [1,2]

2.1 Introduction

Idiopathic pain disorders (IPDs) including fibromyalgia (FM), headache, temporomandibular disorder (TMD), and vestibulodynia (VBD) constitute a significant healthcare problem, affecting over 100 million Americans.[185-191] These disorders occur more frequently in females than males[192] and are chronic in nature, characterized by pain that occurs daily and spans years. While the mechanisms underlying IPDs are poorly understood, emerging evidence indicates a role for adrenergic pathways. Patients exhibit increased levels of catecholamines[131-133] alongside diminished activity of catechol-O-methyltransferase (COMT),[127,128] a ubiquitously expressed enzyme that metabolizes catecholamines to their inactive derivatives.[125] An increase in catecholaminesis is similarly observed in patients with inflammatory conditons such as arthritis and complex regional pain syndrome (CRPS).[193-195] Furthermore, functional variants in the COMT gene that reduce COMT activity[128,196,197] are associated with increased susceptibility to FM,[145,146,198-200] TMD,[141] and experimental pain[141,201] as well as impaired response to treatment.[139,202] It is estimated, based on the frequency of allele variation, that nearly two-thirds of patients with persistent pain disorders possess the low-activity COMT variants.[203,204]

Consistent with clinical disorders, our lab found that administration of the COMT inhibitor OR486 in rodents produces increased hypersensitivity at multiple body sites and alters cognitive-affective behaviors linked to pain (*e.g.,* avoidance of painful heat and bright light).[147,149,150] Pharmacologic studies further revealed that OR486-induced pain is blocked

by administration of the non-selective βAR antagonist propranolol or by combined administration of selective β_2- and β_3AR antagonists.[147,149,150] These results are in line with those from clinical studies, showing that propranolol alleviates pain among FM and TMD patients.[140,151] Collectively, these studies suggest that increased catecholamine levels, resulting from reduced COMT activity, drive pain *via* β_2- and β_3ARs.

β_2- and β_3ARs are G protein-coupled receptors (GPCRs) expressed in peripheral and central regions where they could mediate pain perception and/or processing. β_2ARs are located on peripheral terminals[159-163] and cell bodies[164-166] of primary afferent nociceptors, keratinocytes,[167-169] immune cells,[170-173] and adipocytes[174] in the periphery; and neurons[175,176] and glial cells[177] in the central nervous system. β_3ARs are located on primary afferent nociceptors,[178] adipocytes,[174] and immune cells[171,172] in the periphery and noradrenergic neurons in the brain.[179] Thus, the aim of this study was to determine a site-of-action for COMT-dependent pain. We hypothesized that peripheral, spinal, and/or supraspinal β_2- and β_3ARs contribute to persistent COMT-dependent pain.

To test this hypothesis, we employed a clinically-relevant model of persistent COMT-dependent pain and evaluated responses to mechanical and thermal stimuli in adrenalectomized rats, lacking peripheral epinephrine, and in intact rats receiving continuous delivery of βAR antagonists *via* intraplantar (i.pl.), intrathecal (i.t.), or intracerebroventricular (i.cv.) routes. Potential sexual dimorphism in the contribution of adrenergic systems to persistent COMT-dependent pain was also assessed.

Results demonstrated that male and female rats receiving sustained OR486 exhibited COMT-dependent mechanical and thermal pain, persisting for two-weeks. In contrast, adrenalectomized rats failed to develop OR486-induced pain. Furthermore, i.pl., but not i.t. or i.c.v., administration of the non-selective βAR antagonist propranolol, β_2AR antagonist ICI118,551, or β_3AR antagonist SR59230A blocked OR486-induced pain. These findings demonstrate the importance of peripheral β_2- and β_3ARs in mediating persistent

pain, and suggest that peripherally-acting βAR antagonists may provide an effective treatment option for patients with persistent pain disorders.

2.2 Materials and Methods

2.2.1 Animals

Adult male and female Sprague-Dawley rats (N=24 intact, N=24 adrenalectomized and N=23 sham) were purchased (Charles River Laboratories, Raleigh, NC) for the first set of experiments. For subsequent βAR antagonist experiments, adult male Sprague-Dawley rats (N=111) were bred in-house. Rats weighed between 200 and 400g for all experimental studies. Rats had *ad libitum* access to standard laboratory chow and water. Adrenalectomized rats were provided with saline water (0.9%) to compensate for the loss of sodium in urine due to the absence of aldosterone. All animal procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of North Carolina at Chapel Hill (UNC). Though rodent models of pain only partially correlate with human conditions, rats were chosen for these experiments because an extensive body of literature exists regarding nociceptive pathways and behavior in this species, and because rat pain behavior assays are readily available and well characterized.[7,205,206]

2.2.2 General Experimental Conditions

First, the effects of sustained COMT inhibition on pain were evaluated in intact rats receiving the COMT inhibitor OR486 or vehicle systemically for a two-week period *via* a 2002 Alzet osmotic pump (Durect Corporation, Cupertino, CA). Next, the contribution of peripheral adrenergic systems to persistent OR486-induced pain was evaluated in adrenalectomized rats, lacking peripheral epinephrine, or sham rats receiving OR486 or vehicle systemically for two-week period *via* an osmotic pump. Finally, the contribution of peripheral, spinal and supraspinal βARs to persistent OR486-induced pain was evaluated in separate groups of intact rats receiving i.pl., i.t. or i.c.v.. βAR antagonists alongside systemic

delivery of OR486 or vehicle for a two-week period *via* an osmotic pump. The βAR antagonists were delivered *via* a catheter attached to a separate 2002 Alzet osmotic pump.

Animals were handled and habituated to the experimenter and environment for four days prior to testing. Responses to punctuate mechanical and thermal stimuli were assessed in intact and adrenalectomized animals 1 day prior to and on days 1, 3, 5, 7, 9, 11, and 13 following pump implantation. For βAR antagonist experiments, pain behaviors were assessed one day prior to and on days 2, 4, 6, 8, 10, 12, and 14 following pump implantation. The rest day between surgery and testing allowed animals to fully recover from catheter implantation. On baseline and testing days, rats were habituated to the mechanical and thermal testing environments for ten to fifteen minutes. Though we were unable to eliminate all environmental factors (*e.g.,* season, humidity, noise) from this study, we minimized others (*e.g.,* experimenter consistency, testing time of day, cage density) that were in our control.[207,208] Animals were randomly assigned to groups, were tested by a single, blinded experimenter at a consistent time of day (morning), and were housed with one to two other rats. The primary outcome reported in this study is behavioral changes, in the form of mechanical allodynia, mechanical hyperalgesia, and thermal hyperalgesia, which are described in detail below under their respective subtitles.

2.2.3 Drug Preparation

OR486 (Tocris, Ellisville, MO) was dissolved in a 5:3:2 ratio of dimethylsulfoxide (DMSO), 0.9% saline, and ethanol.[147] For peripheral experiments βAR antagonists propranolol hydrochloride (Tocris, Ellisville, MO), ICI-118,511 (Tocris, Ellisville, MO), and SR59230A (Tocris, Ellisville, MO) were each dissolved in 5:3:2 ratios of DMSO, 0.9% saline, and ethanol. For i.t. and i.c.v. experiments, βAR antagonists were dissolved in 0.9% saline. Drug solutions were injected into pumps, which were placed in 15mL conical tubes containing sterile 0.9% saline and primed overnight in a dry heat bath (Lab Armor, Cornelius, OR) at 37 degrees Celsius. All pumps (other than those for i.t. delivery) were

attached to corresponding catheters prior to priming. Subcutaneous delivery of OR486 was at a constant rate of 15mg/kg/day for a two-week period. Peripheral delivery of propranolol hydrochloride was at 9mg/kg/day, ICI-118,511 was at 1.5mg/kg/day, and SR59230A was at 1.67mg/kg/day. I.t. delivery of propranolol hydrochloride was at 50ug/day for the low dose experiments and 100ug/day for the high dose experiments, ICI-118,511 was at 30ug/day, and SR59230A was at 20ug/day. I.c.v. delivery of propranolol hydrochloride was at 50ug/day for the low dose experiments and 100ug/day for the high dose experiments, ICI-118,511 was at 30ug/day, and SR59230A was at 20ug/day.

2.2.4 Surgical Procedures

For all surgical procedures, rats were anesthetized by isoflurane inhalation (5% induction, 1.5-5% maintenance). Incision sites were shaved and disinfected with ethanol and betadine. Sterile technique was employed throughout the duration of all procedures according to IACUC requirements. Stainless steel wound clips (Braintree Scientific, Braintree, MA) were used to close the wounds.

For systemic delivery of OR486, a small incision was made over the left shoulder blade of the rat. Hemostats were used to create a small subcutaneous pocket, in which the pump was placed.

For i.pl. delivery of βAR antagonists, a modified version of the protocol published by Haddad et al[209] was used. Pumps were attached to a 15cm, Y-shaped, bifurcated 3F silicone catheter (SAI Infusion Technologies, Libertyville, IL). The pump was implanted subcutaneously over the right shoulder blade and a stainless steel 10G X 20cm semi-blunt tip trocar (SAI Infusion Technologies, Libertyville, IL) was used to subcutaneously route the catheter ends to incisions made at either hind paw. The catheter ends were attached to the plantar fascia using 4-0 silk sutures (Oasis Medical, Mettawa, IL).

For i.t. delivery[210] of βAR antagonists, a small incision was made on the nape of the neck, and scissors and hemostats were used to lift muscle and expose the atlanta-occipital

membrane. The membrane was carefully incised using the tip of scissors, causing the escape of cerebral spinal fluid (CSF). A 27.3cm, polyurethane Alzet Short Rat IT Catheter (Durect Corporation, Cupertino, CA) was inserted into the intrathecal space, dorsal to the spinal cord. The other end of the catheter was sutured to surrounding tissue and attached to the osmotic pump, which was subcutaneously implanted over the right shoulder blade. Four animals did not wake up following i.t. surgery. These animals were replaced in future i.t. groups to account for the decrease in sample size.

For i.c.v. delivery[211] of βAR antagonists, pumps were attached to a 38-gauge stainless steel cannula *via* a short vinyl catheter (Alzet Brain Infusion Kit 2, Durect Corporation, Cupertino, CA). The cannula was implanted into the right lateral ventricle (from the bregma: -0.8mm anteroposterior, -1.6mm mediolateral, -5mm dorsoventral) and was cemented to two anchoring screws on the skull. The attached pump was subcutaneously implanted over the right shoulder blade.

2.2.5 Assessment of Behavioral Responses to Mechanical and Thermal Stimuli

Paw withdrawal threshold was assessed using the von Frey up-down method.[212] Nine calibrated and logarithmically spaced von Frey monofilaments (bending forces: 0.40, 0.68, 1.1, 2.1, 3.4, 5.7, 8.4, 13.2, and 15.0g; Stoelting, Wood Dale, IL) were applied to the plantar hind paw. First, the middle filament (3.4g) was applied to the hind paw for three seconds. If the rat responded with a withdrawal, an incrementally lower filament was applied. In the absence of a withdrawal, an incrementally higher filament was applied. A series of six total responses were recorded for each paw. Results were entered into the Paw Flick module within the National Instruments LabVIEW 2.0 software (LabVIEW, Austin, TX), which uses a logarithmic algorithm to determine the gram force value that would elicit paw withdrawal in 50% of trials ($10^{(Xf+k\delta)}/10{,}000$, where X_f = value [in log units] of the final von Frey hair used; k = tabular value of positive and negative responses, and δ = mean difference [in log units] between stimuli). Mechanical allodynia was defined as a heightened

response to a normally innocuous stimulus, as determined by a decrease in paw withdrawal threshold.

Mechanical hyperalgesia was assessed using a 15.0g von Frey filament. This filament was chosen as a normally noxious stimulus, as it has a gram force value well over the 50% withdraw threshold for animals tested in the present study. The filament was applied to the hind paw ten times for a duration of one second, with an interstimulus interval of one second.[147] The number of paw withdrawals (which could range from 0-10) was recorded for each hind paw at each time point. Mechanical hyperalgesia was defined as an increase in the number of paw withdrawals in response to a normally noxious mechanical stimulus.

Thermal hyperalgesia was assessed using the Hargreaves method.[213] Animals were placed in Plexiglass chambers and a radiant beam of light was applied to the hind paw through a glass floor heated to 30 degrees Celsius. Paw withdrawal latencies were recorded in duplicate per paw. If the second latency recorded was not within ±4 seconds of the first, a third measure was recorded. The 2 latencies closest in value were averaged to determine overall latency to withdrawal. Thermal behavioral data is reported in text and figures as the difference in paw withdrawal latency from baseline (Day 0). Thermal hyperalgesia was defined as a decrease in paw withdrawal latency in response to a noxious thermal stimulus.

2.2.6 Statistical Analyses

Sample sizes were selected based on their ability in previous, similarly structured rat studies to accurately demonstrate behavioral differences between groups.[147,149,150] Mechanical allodynia, mechanical hyperalgesia, and thermal hyperalgesia data were analyzed by 2-way analysis of variance (ANOVA for Group X Time). In ANOVA analyses, Groups correspond to the separate groups on the graph of interest, as denoted by different symbols and names (*e.g.,* groups in Figure 1 = Veh and OR486). Post-hoc comparisons were performed using the Bonferroni test, which corrected for multiple comparisons.

Statistical significance was defined as $P<0.05$. All statistical analyses were performed using GraphPad Prism (GraphPad Software, La Jolla, CA).

2.3 Results

2.3.1 Sustained COMT inhibition produces persistent pain

Genetic and pharmacologic alterations resulting in reduced COMT activity are associated with increased experimental pain and likelihood of developing persistent pain disorders. Acute administration of the COMT inhibitor OR486 results in enhanced mechanical and thermal pain in rats.[147] To evaluate the effects of sustained COMT inhibition on pain, responses to mechanical and thermal stimuli were measured in separate groups of rats receiving systemic OR486 (15mg/kg/day) or vehicle over a two-week period. Compared to rats receiving vehicle, those receiving OR486 exhibited mechanical allodynia (group: $p<0.0001$; group x day: $p=0.0043$; Figure 2.1A), mechanical hyperalgesia (group: $p<0.0001$; group x day: $p=0.0109$; Figure 2.1B), and thermal hyperalgesia (group: $p<0.0001$; group x day: $p<0.0001$; Figure 2.1C) beginning on day 1 and lasting throughout the duration of the experiment. Sexual dimorphism was not observed, as both male and female rats developed mechanical allodynia (male group: $p<0.0001$; female group: $p<0.0001$; Figure 2.2A), mechanical hyperalgesia (male group, $p<0.0053$; female group: $p<0.0001$; Figure 2.2B), and thermal hyperalgesia (male group: $p<0.0001$; female group: $p<0.0001$; Figure 2.2C). See Figure 2.2 for all sexual dimorphism data in intact rats.

2.3.2 Adrenalectomized rats fail to develop persistent COMT-dependent pain

Previous work has demonstrated that acute COMT-dependent pain is mediated *via* β_2- and β_3ARs, which are located in peripheral, spinal, and supraspinal regions where they could potentially drive pain transmission. To evaluate the potential contribution of peripheral adrenergic systems to COMT-dependent pain, separate groups of adrenalectomized rats (lacking peripheral epinephrine) or sham surgery rats received systemic OR486

(15mg/kg/day) or vehicle over a two-week period and responses to mechanical and thermal stimuli were measured. Compared to sham rats receiving vehicle, those receiving OR486 developed mechanical allodynia (group: $p<0.0001$; group x day: $p<0.0001$; Figure 2.3A), mechanical hyperalgesia (group: $p<0.0001$; group x day: $p=0.0044$; Figure 2.3B), and thermal hyperalgesia (group: $p=0.0005$; group x day: $p<0.0001$; Figure 2.3C). In contrast, adrenalectomized rats did not develop mechanical allodynia, mechanical hyperalgesia, or thermal hyperalgesia.

Sexual dimorphism was not observed, as both male and female sham rats developed mechanical allodynia (male group: $p<0.0001$; female group: $p<0.0001$; Figure 2.4A), mechanical hyperalgesia (male group: $p=0.0053$; female group: $p<0.0001$; Figure 2.4B), and thermal hyperalgesia (male group: $p<0.0001$; female group: $p<0.0001$; Figure 2.4C). Both male and female adrenalectomized rats failed to develop mechanical allodynia (Figure 2.4D), mechanical hyperalgesia (Figure 2.4E), and thermal hyperalgesia (Figure 2.4F). See Figure 2.4 for all sexual dimorphism data in sham and adrenalectomized rats.

2.3.3 Peripheral βAR antagonist administration prevents the development of persistent COMT-dependent pain

Adrenalectomized rats fail to develop persistent pain following COMT inhibition, suggesting a peripheral adrenergic site of action. To further investigate this hypothesis, pharmacological methods were used to determine the contribution of peripheral, spinal, and supraspinal βARs to persistent COMT-dependent pain (Figure 2.5). First, the contribution of peripheral βARs to mechanical and thermal pain was evaluated in separate groups of rats receiving sustained i.pl. administration of propranolol (9mg/kg/day), ICI-118,551 (1.5mg/kg/day), SR59230A (1.67mg/kg/day), or vehicle alongside sustained systemic administration of OR486 (15mg/kg/day) or vehicle over a two-week period. Peripheral antagonist doses were selected based on the results from a preliminary study that evaluated the ability of three different doses per antagonist to reduce or block COMT-dependent pain.

Compared to rats receiving vehicle, those receiving sustained i.pl. administration of the non-selective βAR antagonist propranolol, the β_2AR antagonist ICI-118,511, or the β_3AR antagonist SR59230A alongside systemic OR486 did not develop mechanical allodynia (group: Figure 2.5A, $p<0.0001$; Figure 2.5D, $p<0.0001$; Figure 2.5G, $p<0.0001$) or mechanical hyperalgesia (group: Figure 2.5B, $p<0.0001$; Figure 2.5E, $p<0.0001$; Figure 2.5H, $p<0.0001$). Rats receiving sustained i.pl. administration of the β_3AR antagonist SR59230A also did not develop OR486-induced thermal hyperalgesia (group: Figure 2.5I, $P<0.0001$). In contrast, rats receiving propranolol (Figure 2.5C) or ICI-118,551 (Figure 2.5F) alongside OR486 exhibited a 15% decrease in paw withdrawal latency from baseline, similar to rats receiving vehicle. Animals receiving sustained i.pl. administration of βAR antagonists alongside systemic vehicle failed to develop mechanical allodynia (Figure 2.10A), mechanical hyperalgesia (Figure 2.10B), or thermal hyperalgesia (Figure 2.10C). See Figure 2.10 for control data demonstrating no effect of antagonists on pain irrespective of administration route.

2.3.4 Intrathecal βAR antagonist administration does not alter persistent COMT-dependent pain

Next, the contribution of spinal βARs to mechanical and thermal pain was evaluated in separate groups of rats receiving sustained i.t. administration of propranolol (50ug/day), ICI-118,551 (30ug/day), SR59230A (20ug/day), or vehicle alongside sustained systemic administration of OR486 (15mg/kg/day) or vehicle over a two-week period (Figure 2.6). Intrathecal delivered antagonist doses were selected based on their ability to block hypersensitivity or pain-relevant behaviors in other rat models when administered i.t..[214-216] Similar to animals receiving vehicle, those receiving sustained i.t. administration of the non-selective βAR antagonist propranolol, the β_2AR antagonist ICI-118,511, or the β_3AR antagonist SR59230A alongside systemic OR486 exhibited mechanical allodynia (group: Figure 2.6A, $p<0.0001$; Figure 2.6D, $p<0.0001$; Figure 2.6G, $p<0.0001$), mechanical

hyperalgesia (group: Figure 2.6B, p=0.0002; Figure 2.6E, p<0.0001; Figure 2.6H, p=0.0018), and thermal hyperalgesia (group: Figure 2.6C, p<0.0001; Figure 2.6F, p<0.0001; Figure 2.6I, p<0.0001). Animals receiving sustained i.t. administration of βAR antagonists alongside systemic vehicle failed to develop mechanical allodynia (Figure 2.10D), mechanical hyperalgesia (Figure 2.10E), or thermal hyperalgesia (Figure 2.10F). Animals receiving SR59230A alongside vehicle did exhibit transient elevations in paw withdrawal threshold on days 2 (Veh/Veh 4.47±0.63 vs Veh/SR 10.80±3.26, mean±SEM) and 10 (Veh/Veh 3.50±0.73 vs Veh/SR 10.97±3.13) likely due to higher baseline values (Veh/Veh 4.76±0.55 vs Veh/SR 8.54±2.59) and increased inter-group variability as compared to control animals (Figure 2.10D).

To confirm that i.t. βAR antagonists were unable to block OR486-induced pain, we performed a duplicate set of experiments using a higher dose of the non-selective βAR antagonist propranolol (100ug/day). Similar to the original dose, i.t. administration of the higher dose did not block OR486-induced mechanical allodynia (group: p<0.0001; Figure 2.7A), mechanical hyperalgesia (group: p=0.0011; Figure 2.7B), or thermal hyperalgesia (group: p<0.0001; Figure 2.7C). See Figure 2.7 for all i.t. high dose propranolol data.

2.3.5 Intracerebroventricular βAR antagonist administration does not alter persistent COMT-dependent pain

Finally, the contribution of supraspinal βARs to mechanical and thermal pain was evaluated in separate groups of rats receiving sustained i.c.v. administration of propranolol (50ug/day), ICI-118,551 (30ug/day), SR59230A (20ug/day), or vehicle alongside sustained systemic administration of OR486 (15mg/kg/day) or vehicle over a two-week period (Figure 2.8). I.c.v. antagonist doses were selected based on their ability to block pain or related behaviors in other rat models.[214-216] Similar to animals receiving vehicle, those receiving sustained i.c.v. administration of the non-selective βAR antagonist propranolol, the β_2AR antagonist ICI-118,511, or the β_3AR antagonist SR59230A alongside systemic OR486

exhibited mechanical allodynia (group: Figure 2.8A, $p<0.0001$; Figure 2.8D, $p<0.0001$; Figure 2.8G, $p<0.0001$), mechanical hyperalgesia (group: Figure 2.8B, $p<0.0001$; Figure 2.8E, $p<0.0001$; Figure 2.8H, $p<0.0001$), and thermal hyperalgesia (group: Figure 2.8C, $p<0.0001$; Figure 2.8F, $p<0.0001$; Figure 2.8I, $p<0.0001$). Animals receiving sustained i.c.v. administration of βAR antagonists alongside systemic vehicle failed to develop mechanical allodynia (Figure 2.10G), mechanical hyperalgesia (Figure 2.10H), or thermal hyperalgesia (Figure 2.10I). Animals receiving SR59230A alongside vehicle did exhibit transient elevations in paw withdrawal frequency on days 2 (Veh/Veh 1.88±0.40 vs Veh/SR 4.62±0.86) and 8 (Veh/Veh 2.00±0.46 vs Veh/SR 5.00±1.20), likely due to increased inter-group variability as compared to control animals (Figure 2.10H).

To confirm that i.c.v. βAR antagonists are unable to block OR486-induced pain, we performed a duplicate set of experiments using a higher dose of the non-selective βAR antagonist propranolol (100ug/day). Similar to the original dose, i.c.v. administration of the higher dose did not block OR486-induced mechanical allodynia (group: $p<0.0001$; Figure 2.9A), mechanical hyperalgesia (group: $p<0.0001$; Figure 2.9B), or thermal hyperalgesia (group: $p<0.0001$; Figure 2.9C). See Figure 2.9 for all i.c.v. high dose propranolol data.

2.4 Discussion

Though the mechanisms underlying IPDs are not well described, emerging evidence suggests a role for adrenergic pathways. Employing a rodent model of sustained COMT inhibition that mimics abnormalities in catecholamine signaling observed in patients with these disorders, we demonstrate that COMT-dependent pain is mediated *via* peripherally, but not spinally or supraspinally, located β_2- and β_3ARs.

In previous studies, we established a causal link between low COMT and pain. We demonstrated that a single injection of the COMT inhibitor OR486 produces mechanical and thermal pain, similar to that produced by intraplantar carrageenan. Subsequent

pharmacological studies further demonstrated that the development of acute OR486-induced pain requires activation of β_2- and β_3ARs.[147,149] Within hours, administration of OR486 results in increased circulating levels of nitric oxide (NO) and the pro-inflammatory cytokines tumor necrosis factor-α (TNFα), interleukin 1β (IL-1β), interleukin 6 (IL-6), and chemokine (C-C motif) ligand 2 (CCL2),[149]which are nociceptive transmitters implicated in persistent pain. Individuals with FM, headache, and TMD exhibit increased levels of these molecules,[32,217-219] which elicit pain by reducing nociceptor firing thresholds.[220-230] NO and pro-inflammatory cytokines also elicit pain by working synergistically to potentiate one another's biosynthesis, as observed in the OR486 model.[149]

Here, we utilized a more clinically-relevant model of sustained COMT inhibition, characterized by enhanced sensitivity to noxious stimuli and altered pain-relevant cognitive-affective behaviors that persist over a two-week period, to determine the site-of-action whereby βARs mediate persistent COMT-dependent pain. The contribution of peripheral adrenergic systems was first examined in adrenalectomized rats. We found that, compared to sham surgery rats, adrenalectomized rats lacking peripheral epinephrine fail to develop OR486-induced mechanical and thermal pain. This finding is in line with those from previous studies showing that adrenalectomized rats have blunted pain following formalin administration[231] or chronic constriction injury.[232] Together, these results suggest that peripherally circulating catecholamines contribute to the transmission of pain in models of inflammatory and neuropathic pain as well as persistent pain disorders. This conclusion is further supported by studies that have demonstrated increased urinary catecholamines in patients with myofacial pain[132] and increased circulating plasma catecholamines in women with FM.[131] Of note, adrenalectomy also results in a reduction of circulating corticosterone levels.[233,234] Increased corticosterone levels following stress[235] or nerve injury[236,237] have been implicated in analgesia, and pronociception, respectively. Thus, future experiments examining peripheral catecholamines should utilize adrenal medullectomized animals or

should provide supplemental corticosterone to adrenalectomized animals to rule out corticosterone-mediated effects.

As previous preclinical and clinical studies have reported sex-specific differences in COMT-related phenotypes[238-242] and as males and females exhibit different COMT expression patterns,[243,244] we examined the contribution of peripheral adrenergic systems to COMT-dependent pain in both sexes. Counter to our expectation, male and female rats exhibited a comparable increase in mechanical and thermal pain following sustained systemic OR486 administration, which was blocked by suppressing peripheral adrenergic tone. Despite these findings, no single study can rule out the possibility of sex-specific effects, so we recommend that future studies and clinical applications related to COMT-dependent pain should continue to consider possible sex-specific effects.

The independent contribution of peripheral, spinal, and supraspinal βARs to persistent COMT-dependent pain were next examined in separate groups of intact rats receiving targeted delivery of the non-selective βAR antagonist propranolol, the β_2AR antagonist ICI-118,551, or the β_3AR antagonist SR59230A alongside systemic OR486. We found that peripheral, but not spinal or supraspinal, administration of propranolol, ICI-118,511, or SR59230A blocked the development of OR486-induced pain throughout the duration of the testing period. While all three antagonists blocked the development of mechanical pain, only SR59230A blocked the development of thermal pain. These findings significantly extend those from acute COMT inhibition studies,[147,149] demonstrating that peripheral β_2- and β_3ARs both contribute to the development of persistent mechanical pain, while peripheral β_3ARs independently contribute to the development of persistent thermal pain following sustained COMT inhibition.

The peripheral contribution of β_2ARs to pain is in line with results from previous studies demonstrating that epinephrine activates β_2ARs located on the peripheral terminals of primary afferent nociceptors, increasing their excitability and producing a hyperalgesic

state.[159-163] Also, elevated plasma norepinephrine activates β_2ARs to promote visceral hypersensitivity.[162] In humans, variants of the β_2AR gene known to influence receptor expression are associated with increased risk of TMD.[245]

The contribution of peripheral β_3ARs to persistent pain is more novel. Peripherally expressed β_3ARs are known for their ability to regulate norepinephrine-induced changes in metabolism and thermoregulation.[157] In 2010, it was discovered that β_3ARs are expressed on primary afferent nociceptors, where they drive norepinephrine-induced ATP release and contribute to neuropathic pain.[246] Recently, β_3ARs have also been shown to mediate formalin-induced TMJ pain.[247] In contrast to acute COMT-dependent thermal pain, which requires coincident activation of both β_2- and β_3ARs,[147] persistent COMT-dependent thermal pain requires independent activation of peripheral β_3ARs. Unlike most GPCRs, including β_2ARs, β_3ARs do not undergo desensitization after agonist stimulation.[248,249] Thus, β_3ARs are uniquely positioned to stimulate downstream effectors for prolonged periods of time.

In addition to their location on primary afferent nociceptors, β_2- and β_3ARs are expressed on numerous peripheral cell types where they could potentially mediate pain, including: immune cells involved in adaptive responses (T-cells, mast cells, and macrophages), adipocytes, keratinocytes, and satellite glia. T-cells, mast cells, and macrophages are immune cells in the periphery that express βARs and, following their activation by epinephrine or norepinephrine, orchestrate inflammatory responses. Increased catecholamine levels following stress or pharmacologic manipulation lead to activation of T-cells, increased expression of β_2- and β_3ARs,[173] and production of IL-1, IL-6, and CCL2.[250] T-cell infiltration in the spinal dorsal horn of adult rats has been shown to contribute to hypersensitivity following nerve injury.[251,252] In line with these findings, patients with FM have more activated T-cells circulating in blood compared to healthy controls.[253] Epinephrine activates mast cells and stimulates release of IL-1β, IL-6, and other pro-inflammatory cytokines in a β_2AR-dependent manner.[170] Increased activation of mast cells has been

observed in numerous persistent pain disorders, including FM, headache, vestibulodynia, and irritable bowel syndrome.[254-259] Agonist activation of β_2ARs expressed on macrophages *in vitro* results in activation of intracellular kinases and release of IL-6. Further, sustained systemic administration of epinephrine in mice results in β_2AR-mediated increases in macrophage activation and IL-6 production.[171,172]

Adipocytes are cells in the periphery that express both β_2- and β_3ARs and specialize in storing energy as fat.[174] They also interface with immune cells to regulate inflammatory responses.[260] Notably, adipocytes produce 30% of the IL-6 circulating in the body[261] and studies have shown that activation of β_3ARs on adipocytes produces a robust increase in IL-6 levels in plasma[262] as well as in TNFα,[263] CCL2,[264] and NO[265] levels in vitro.

Keratinocytes and satellite glial cells reside near the peripheral terminals and cell bodies, respectively, of primary afferent nociceptors. While a direct link between βAR activation on these cell types and pain has yet to be established, catecholamine-induced activation of keratinocyte β_2ARs results in increased intracellular kinase activation and IL-6 release.[167-169] Similarly, activation of satellite glia by catecholamines results in βAR-mediated increases in intracellular cyclic nucleotides that facilitate neuronal-glial communication.[266]

Collectively, these findings demonstrate the importance of β_2- and β_3ARs located on immunoregulatory cells in the periphery to persistent COMT-dependent pain, accounting for clinical observations that βAR antagonists provide pain relief for patients with idiopathic pain disorders such as FM and TMD,[140,151,267] as well as inflammatory conditions such as arthritis, rosacea, and CRPS.[154,268-270] While these findings seem inconsistent with the ability of antidepressants to alleviate persistent pain by increasing synaptic levels of catecholamines, it is important to note that the analgesic effect of antidepressants is associated with descending inhibition of pain *via* actions at α_2ARs or D_2DARs in the spinal dorsal horn.[271,272] Thus, catecholamines can exert divergent influences on nociception as a function of localization and net influence on neuronal excitability. Future studies are required to identify

the specific cell type(s) in the periphery that express βARs and, upon activation, release pro-inflammatory molecules that initiate persistent hypersensitivity. By determining where, when and how β_2- and β_3ARs and their downstream effectors mediate COMT-dependent pain, the field will better understand the diverse nature of catecholamine signaling so that patients suffering from disorders resulting from reduced COMT and/or elevated catecholamines receive the most relevant treatments.

While the studies herein utilized a clinically-relevant rodent model of sustained COMT inhibition, additional mechanistic studies will implement a COMT-/- mouse model to more accurately represent the endogenously low levels of COMT activity observed in pain patients. Future studies are also necessary to elucidate the specific cell signaling pathways responsible for the initiation and maintenance of β_2- and β_3AR-mediated pain. Finally, clinical studies are required to evaluate the efficacy of peripheral β_2- and β_3AR antagonist therapy in patients with persistent pain disorders and related conditions.

In conclusion, we utilized a clinically-relevant animal model that portrays characteristics of patients with persistent pain disorders to demonstrate that both male and female rats are susceptible to the development of persistent COMT-dependent pain, which is mediated *via* peripherally located β_2- and β_3ARs. These findings suggest that peripheral β_2- and β_3AR antagonist therapy may be an effective option for the treatment of persistent pain disorders as well as those with overlapping peripheral β-adrenergic mechanisms (*e.g.,* complex regional pain syndrome[273]).

2.5 Acknowledgements

The authors thank SAI-Infusion Technologies for their assistance with catheter design.

2.6 Footnotes

[1]Reprinted from *Anesthesiology,* Ciszek BP, O'Buckley SC, Nackley A, Persistent catechol-O-methyltransferase is initiated by peripheral adrenergic receptors, *Article In Press*, Copyright (2016); with permission from Elsevier.[274] Final Text Available Online at: http://www.ncbi.nlm.nih.gov/pubmed/26950706.

[2] This work was funded by R01 NS072205 to A.N and P01 NS045685 to A.N (NIH/NINDS, Bethesda, MD, USA 20892).

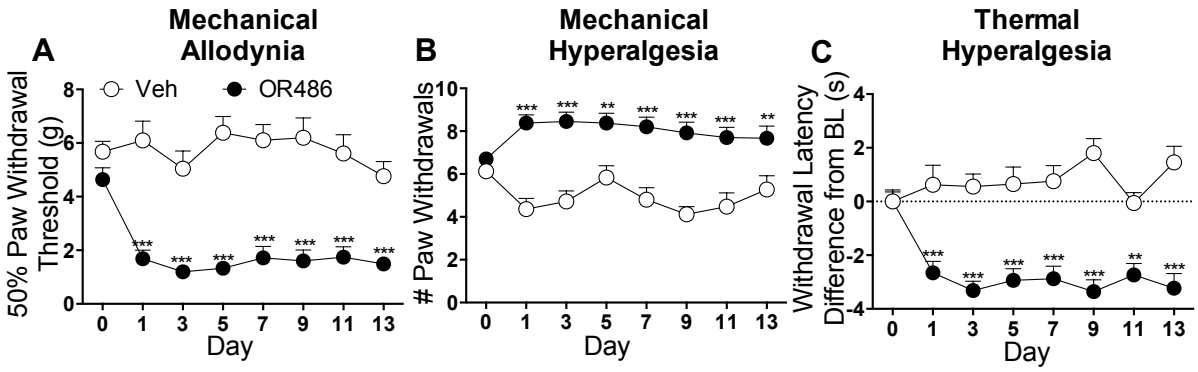

Figure 2.1 Sustained administration of the COMT inhibitor OR486 leads to mechanical and thermal pain. Compared to vehicle, sustained systemic OR486 administration produces **(A)** mechanical allodynia, **(B)** mechanical hyperalgesia, and **(C)** thermal hyperalgesia. N=12 (6M, 6F) per group. Data are expressed as mean ± SEM. ***P<0.001, **P<0.01 different from vehicle. Abbreviations: Baseline (BL), Vehicle (Veh).

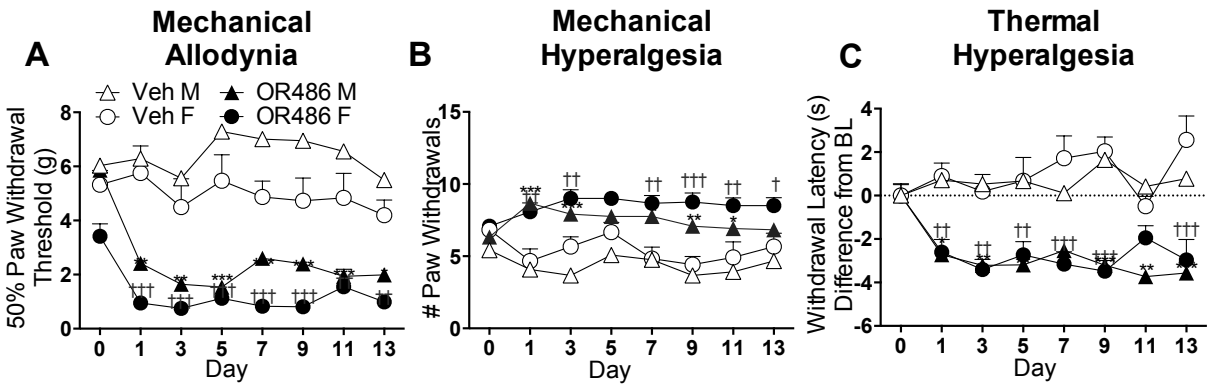

Figure 2.2 Sustained administration of the COMT inhibitor OR486 leads to increased mechanical and thermal pain in male and female rats. Compared to vehicle, sustained OR486 administration produces **(A)** mechanical allodynia, **(B)** mechanical hyperalgesia, and **(C)** thermal hyperalgesia in males and females. N=6 per group. Data are expressed as mean ± SEM. ***P<0.001, **P<0.01, *P<0.05 different from Veh/M; †††P<0.001, ††P<0.01, †P<0.05 different from Veh/F. Abbreviations: Baseline (BL), catechol-o-methyltransferase (COMT), Female (F), Male (M), Vehicle (Veh).

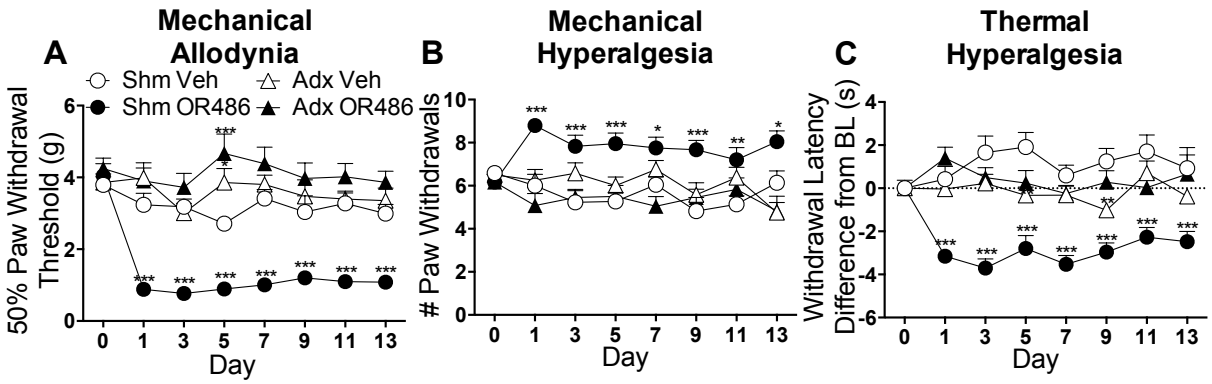

Figure 2.3 Adrenalectomized rats fail to develop OR486-induced pain. In Shm, but not Adx animals, sustained systemic OR486 administration produces **(A)** mechanical allodynia, **(B)** mechanical hyperalgesia, and **(C)** thermal hyperalgesia. N=11 (5M, 6F) for Shm/Veh and N=12 (6M, 6F) for all other groups. Data are expressed as mean ± SEM. ***P<0.001, **P<0.01, *P<0.05 different from Shm/Veh. Abbreviations: Adrenalectomized (Adx), Baseline (BL), Sham (Shm), Vehicle (Veh).

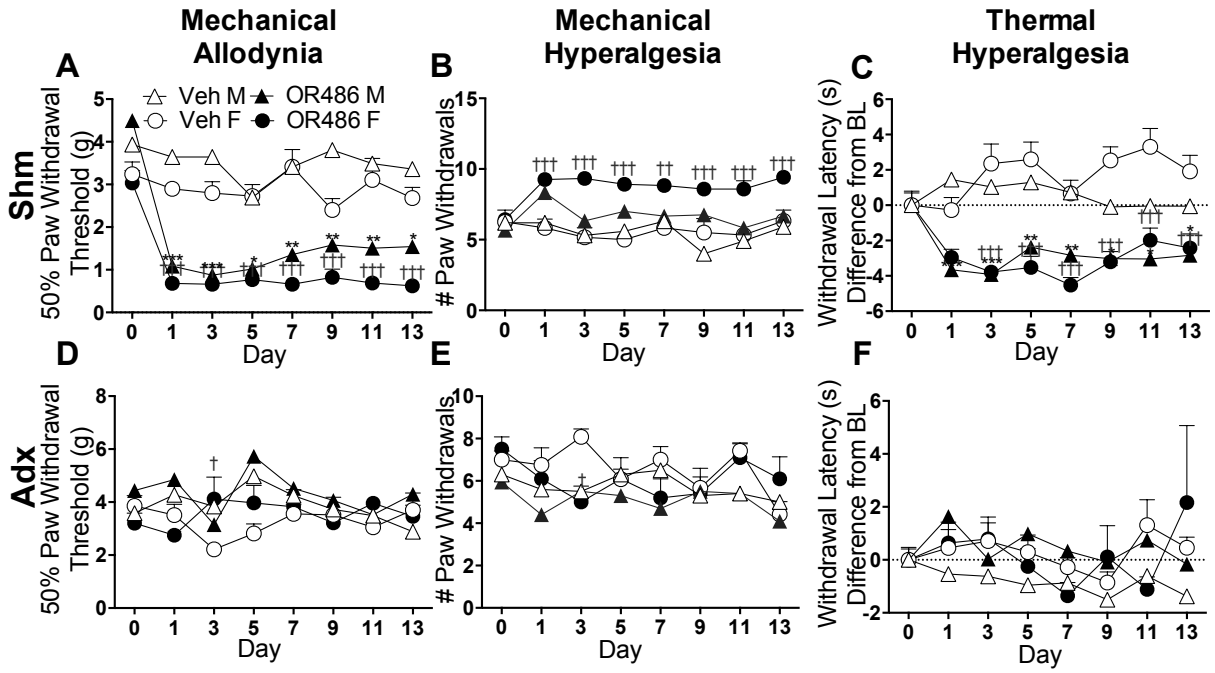

Figure 2.4 Male and female adrenalectomized rats fail to develop OR486-induced pain. In Shm animals, sustained systemic OR486 administration produces **(A)** mechanical allodynia, **(B)** mechanical hyperalgesia, and **(C)** thermal hyperalgesia. In contrast, Adx animals fail to develop OR486-induced **(D)** mechanical allodynia, **(E)** mechanical hyperalgesia, or **(F)** thermal hyperalgesia. N=5-6 per group. Data are expressed as mean ± SEM. ***P<0.001, **P<0.01, *P<0.05 different from Veh/M; †††P<0.001, ††P<0.01, †P<0.05 different from Veh/F. Abbreviations: Adrenalectomized (Adx), Baseline (BL), Female (F), Male (M), Sham (Shm), Vehicle (Veh).

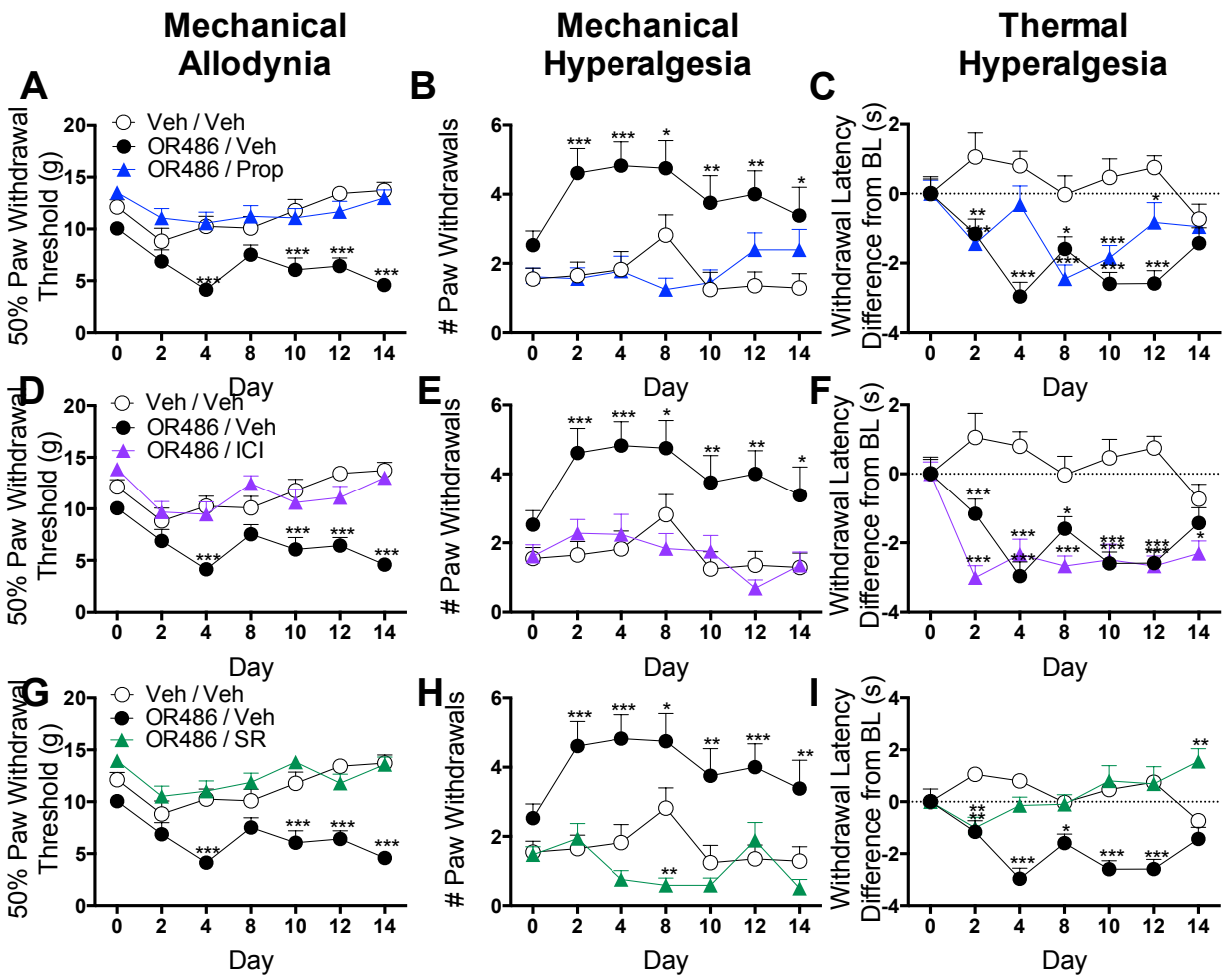

Figure 2.5 Peripheral administration of βAR antagonists blocks OR486-induced pain. Peripheral delivery of the non-selective βAR antagonist propranolol alongside sustained systemic OR486 administration prevents **(A)** mechanical allodynia and **(B)** mechanical hyperalgesia, but does not alter **(C)** thermal hyperalgesia. Similarly, peripheral delivery of the β_2AR antagonist ICI-118,551 alongside sustained systemic OR486 administration prevents **(D)** mechanical allodynia and **(E)** mechanical hyperalgesia, but does not alter **(F)** thermal hyperalgesia. Finally, peripheral delivery of the β_3AR antagonist SR59230A alongside sustained systemic OR486 administration prevents **(G)** mechanical allodynia, **(H)** mechanical hyperalgesia, and **(I)** thermal hyperalgesia. N=9 per group. Data are expressed as mean ± SEM. ***P<0.001, **P<0.01, *P<0.05 different from Veh/Veh. Abbreviations: Baseline (BL), Beta-Adrenergic Receptor (βAR), ICI-118,551 (ICI), propranolol (Prop), SR59230A (SR), Vehicle (Veh).

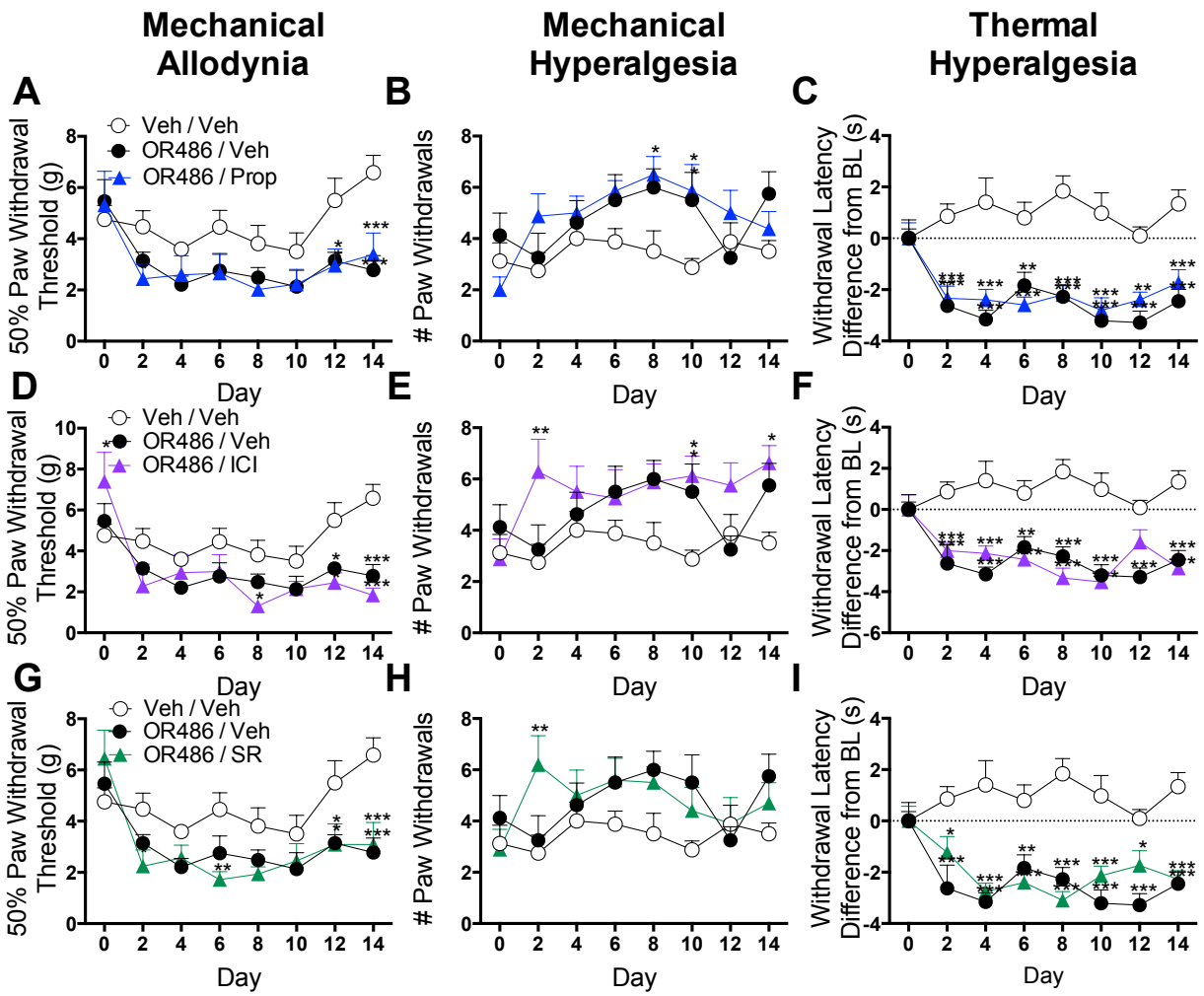

Figure 2.6 Intrathecal administration of βAR antagonists does not alter OR486-induced pain. Intrathecal delivery of the non-selective βAR antagonist propranolol **(A-C)**, β_2AR antagonist ICI-118,551 **(D-F),** or the β_3AR antagonist SR59230A **(G-I)** alongside sustained systemic OR486 administration does not alter mechanical or thermal sensitivity. N=4 per group. Data are expressed as mean ± SEM. ***P<0.001, **P<0.01, *P<0.05 different from Veh/Veh. Abbreviations: Baseline (BL), Beta-Adrenergic Receptor (βAR), ICI-118,551 (ICI), propranolol (prop), SR59230A (SR), Vehicle (Veh).

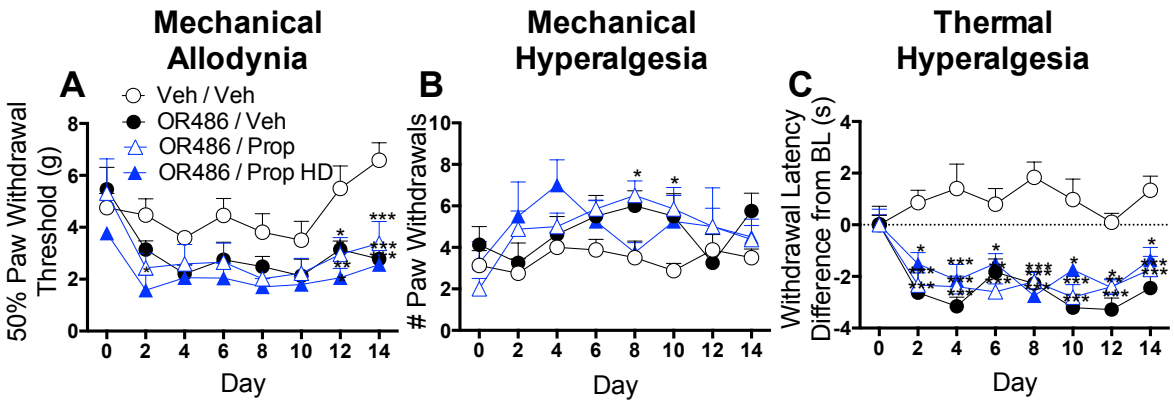

Figure 2.7 Intrathecal administration of high dose propranolol does not alter OR486-induced pain. Intrathecal delivery of a high dose of the non-selective βAR antagonist propranolol (100ug/day) alongside sustained OR486 administration does not alter **(A)** mechanical allodynia, **(B)** mechanical hyperalgesia, or **(C)** thermal hyperalgesia. N=4 for Veh/Veh, OR486/Veh, and OR486/Prop; and N=2 for OR486/Prop HD. Data are expressed as mean ± SEM. ***P<0.001, **P<0.01, *P<0.05 different from Veh/Veh. Abbreviations: Baseline (BL), Beta-Adrenergic Receptor (βAR), High Dose (HD), Propranolol (Prop), Vehicle (Veh).

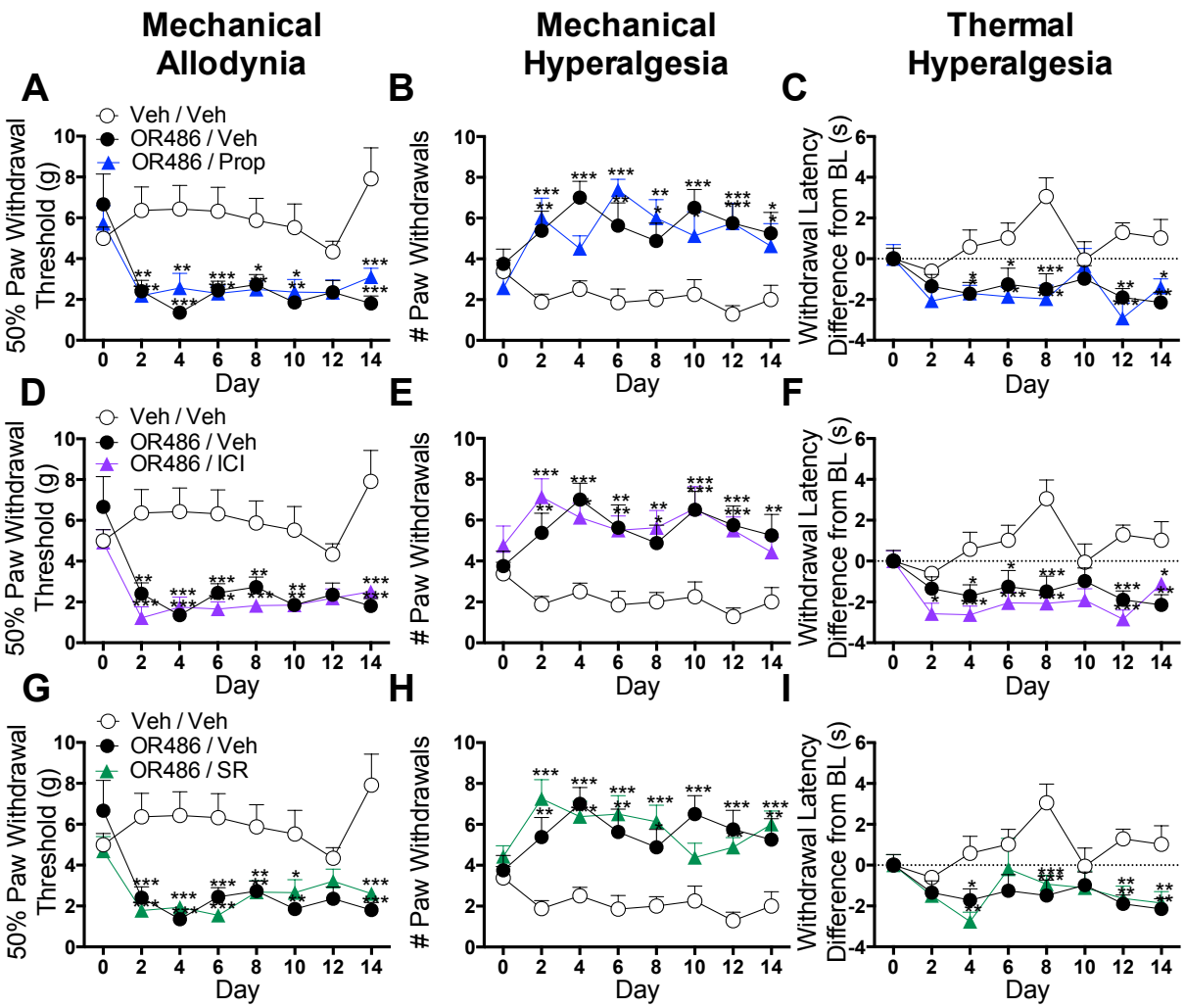

Figure 2.8 Intracerebroventricular administration of βAR antagonists does not alter OR486-induced pain. Supraspinal delivery of the non-selective βAR antagonist propranolol **(A-C)**, β_2AR antagonist ICI-118,551 **(D-F),** or the β_3AR antagonist SR59230A **(G-I)** alongside sustained systemic OR486 administration does not alter mechanical or thermal sensitivity. N=4-5 per group. Data are expressed as mean ± SEM. ***P<0.001, **P<0.01, *P<0.05 different from Veh/Veh. Abbreviations: Baseline (BL), Beta-Adrenergic Receptor (βAR), ICI-118,551 (ICI), propranolol (prop), SR59230A (SR), Vehicle (Veh).

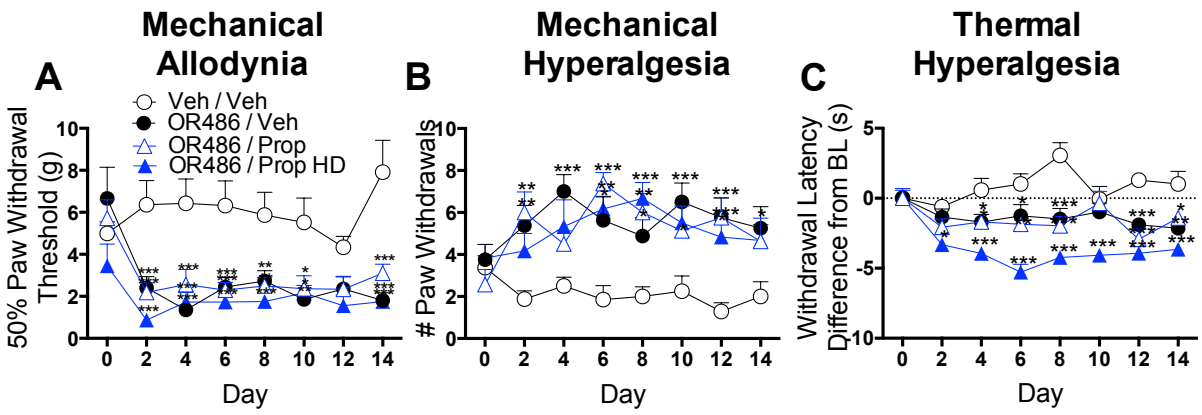

Figure 2.9 Intracerebroventricular administration of high dose propranolol does not alter OR486-induced pain. Intracerebroventricular delivery of a high dose of the non-selective βAR antagonist propranolol (100ug/day) alongside sustained OR486 administration does not alter **(A)** mechanical allodynia, **(B)** mechanical hyperalgesia, or **(C)** thermal hyperalgesia. N=3-4 per group. Data are expressed as mean ± SEM. ***P<0.001, **P<0.01, *P<0.05 different from Veh/Veh. Abbreviations: Baseline (BL), Beta-Adrenergic Receptor (βAR), High Dose (HD), Propranolol (prop), Vehicle (Veh).

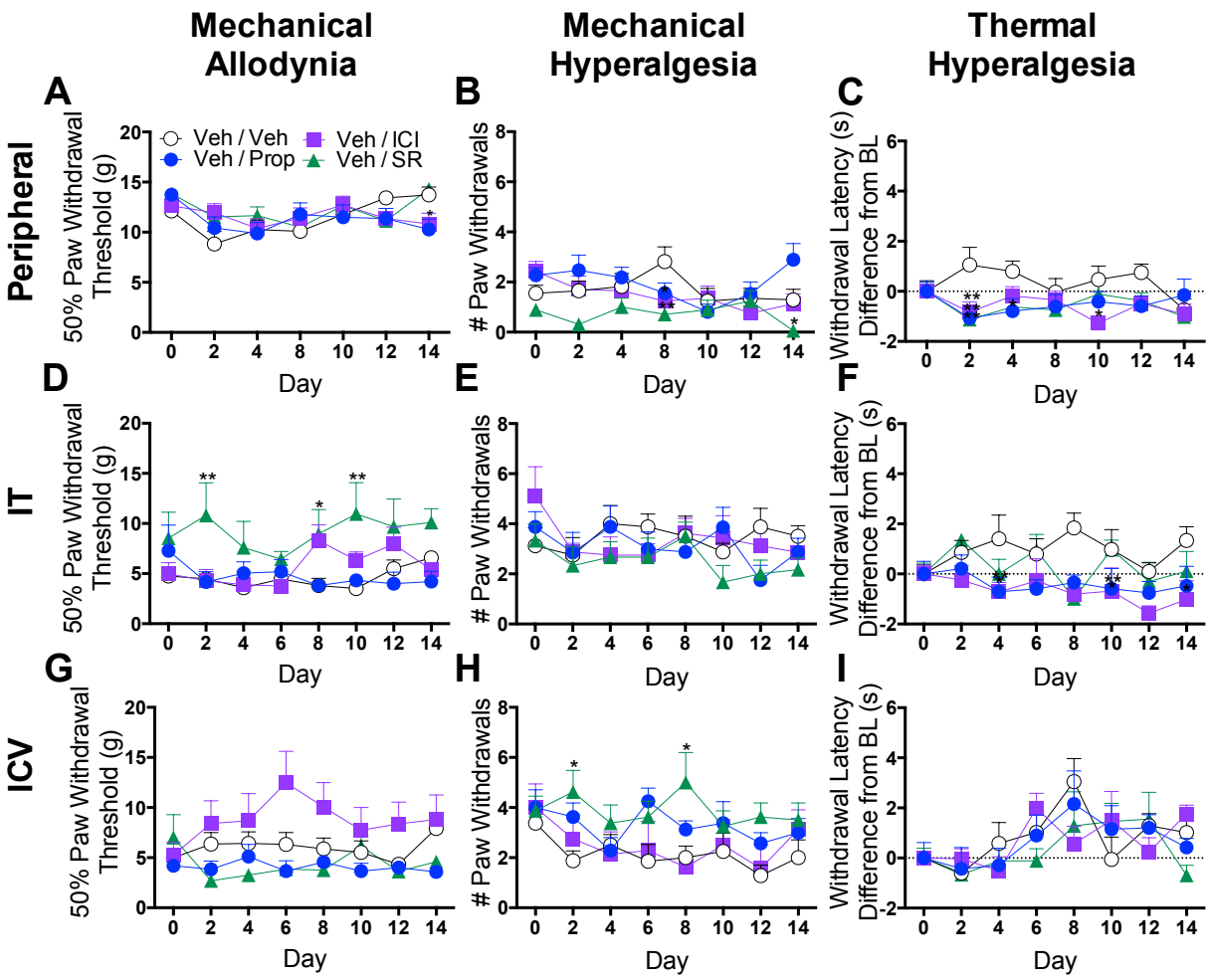

Figure 2.10 Sustained peripheral, intrathecal, or intracerebroventricular administration of βAR antagonists, in the absence of systemic administration of OR486, does not alter pain. (A-C) Peripheral, **(D-F)** i.t., or **(G-I) i**.c.v. delivery of the non-selective βAR antagonist propranolol, β_2AR antagonist ICI-118,551, or β_3AR antagonist SR59230A does not alter **(A,D,G)** mechanical allodynia, **(B,E,H)** mechanical hyperalgesia, or **(C,F,I)** thermal hyperalgesia. N=8-9 per group. **P<0.01, *P<0.05 different from Veh/Veh. Abbreviations: Baseline (BL), Beta-Adrenergic Receptor (βAR), ICI-118,551 (ICI), intracerebroventricular (i.c.v.), intrathecal (i.t.), propranolol (prop), SR59230A (SR), Vehicle (Veh).

CHAPTER 3

MECHANICAL AND THERMAL PAIN IN CATECHOL-O-METHYLTRANSFERASE KNOCKOUT MICE IS NOT MAINTAINED BY BETA-ADRENERGIC RECEPTORS

3.1 Introduction

Idiopathic pain disorders (IPDs), such as fibromyalgia (FM), temporomandibular disorder (TMD) and vestibulodynia (VBD) are characterized by pain that often occurs daily and spans years. These conditions affect nearly 100 million Americans and cost the US economy over $600 billion annually,[191] thus represent a significant healthcare problem. Though the mechanisms underlying IPDs are not well understood, adrenergic pathways are known to play an important role. Patients exhibit increased levels of catecholamines[131-133] along with decreased activity of catechol-O-methyltransferase (COMT),[127,128] a ubiquitously expressed enzyme that metabolizes catecholamines to their inactive derivatives.[125] Functional variants in the COMT gene, which reduce COMT activity,[128,196,197] are associated with increased experimental pain[141,201] as well as risk of developing an IPD.[141,145,146,198-200] It is estimated, based on the frequency of allele variation, that nearly two-thirds of patients with persistent idiopathic pain possess the low-activity COMT variant.[203,204] Refer to Chapter 2.1 for more information regarding the association between COMT and pain.

As demonstrated in Chapter 2, the initiation of COMT-dependent pain is mediated *via* peripherally located β_2- and β_3- adrenergic receptors (βARs). This was demonstrated using a pharmacological model of COMT-dependent pain in rats. Male and female rats receiving sustained administration of the COMT inhibitor OR486 exhibited mechanical and thermal pain that persisted for the duration of the study. Adrenalectomized rats, lacking

peripheral adrenal catecholamines, failed to develop OR486-induced pain. Furthermore, peripheral, but not spinal or supraspinal, coadministration of β_2- and β_3AR antagonists were able to block OR486-induced pain.[274] These findings demonstrate the importance of peripheral β_2- and β_3ARs in initiating persistent COMT-dependent pain.

Though the previous studies described in Chapter 2 utilize a clinically relevant rodent model of sustained COMT inhibition, OR486 delivery was limited to two weeks.[274] One way to more closely model the etiology of persistent pain due to low activity COMT alleles is through use of genetic knockdown of COMT. In the present study, we utilize a COMT-/- mouse model in order to observe pain behaviors in animals that express endogenously low COMT activity levels throughout their lifespan. The aim was to determine whether pain response varied according genotype and if so, whether genetic COMT-dependent pain was mediated *via* peripheral βARs. We hypothesized that, similar to rats receiving the COMT inhibitor, COMT-/- mice would exhibit mechanical and thermal pain mediated *via* peripherally located β_2- and β_3ARs.

To test this hypothesis, we evaluated responses to mechanical and thermal stimuli in female and male COMT+/+, +/-, and -/- mice. As predicted, male and female COMT -/- mice exhibited mechanical and thermal pain that persisted for the duration of the study. Surprisingly, COMT+/- mice did not exhibit pain in response to mechanical and thermal stimuli. Next, we coadministered β_2- and β_3AR antagonists peripherally *via* a bifurcated intraplantar catheter to COMT+/+ and -/- mice over a 2-week period. Unlike the pharmacologic rat model, peripherally delivered β_2- and β_3AR antagonists were unable to block pain in the -/- mice. These findings demonstrate that though β_2- and β_3ARs play a vital role in the initiation of COMT-dependent pain, they do not contribute to the maintenance of established COMT-dependent pain. This is important knowledge for clinicians who treat persistent pain associated with abnormalities in catecholamine signaling.

3.2 Materials and Methods

3.2.1 Animals

The Gogos laboratory at Columbia University (New York, NY) provided adult male and female COMT+/- mice, which expectedly display disruption of the *COMT* gene.[238] Heterozygote mice were intercrossed in-house to create wildtype (COMT+/+; N=24), heterozygote (COMT+/-; N=13), and knockout (COMT-/-; N=26) mice for the present study. Mice weighed between 15 and 30g for all experimental studies. Mice had *ad libitum* access to standard laboratory chow and water. All animal procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of North Carolina at Chapel Hill (UNC). Though rodent models of pain only partially correlate with human conditions, mice were chosen for these experiments because an extensive body of literature exists regarding nociceptive pathways and behavior in this species, and because mouse pain behavior assays are readily available and well characterized.[7,205,206] Further, COMT-/- mice allow us to study COMT-dependent pain in animals that have had COMT deficiencies throughout development. This mimics inherent COMT deficiencies observed in patients with persistent pain.[141,145,146,198-200]

3.2.2 General Experimental Conditions

As previous work[274] (See Chapter 2) in our lab has demonstrated a role for β_2- and β_3-ARs in the initiation of COMT-dependent pain, the present study aimed to investigate whether these receptors are required for the maintenance of established COMT-dependent pain, using COMT-/- mice. First, responses to punctuate mechanical and thermal stimuli were measured in COMT+/+, +/-, and -/- mice to determine if knockdown of the COMT gene leads to enhanced pain behaviors in mice, as pharmacologic COMT inhibition does in the rat model (Chapter 2).[274] Next, the contribution of peripheral β_2- and β_3-ARs to persistent COMT-dependent pain was evaluated in COMT+/+ and -/- mice receiving simultaneous

intraplantar (i.pl.) delivery of the β_2AR antagonist ICI-118,511 and the β_3-AR antagonist SR59230A. The antagonists were delivered to the hindpaws *via* a subcutaneous (s.c.) bifurcated catheter attached to a 1002 Alzet osmotic pump. The contribution of β_2- and β_3-ARs to persistent COMT-dependent pain were further evaluated in COMT+/+ and -/- mice by acute intraperitoneal (i.p.) injection of a bolus dose of ICI-118,511 and SR59230A.

Animals were handled and habituated to the experimenter and environment for 4 days prior to testing. For the persistent study, responses to punctuate mechanical stimuli were assessed in mice 1 day prior to and on days 1, 3, 5, 7, 9, 11, and 13, and responses to heat stimuli were measured prior to and on days 1, 7, and 13, following pump implantation. For the acute study, responses to punctuate mechanical stimuli were assessed in mice prior to and 30, 60, 90, and 120 minutes, and responses to heat stimuli were measured prior to and 60 and 120 minutes, following injection. Thermal behaviors were not assessed at every timepoint to avoid the possibility of hypersensitivity of the hindpaws as a result of this relatively invasive thermal pain measurement. On baseline and testing days, mice were habituated to the mechanical and thermal testing environments for 15-30 minutes. Animals were randomly assigned to groups, housed with 1-2 other mice, and tested by a single, blinded experimenter at a consistent time in the morning. The primary outcome reported in this study is behavioral changes, in the form of mechanical allodynia, mechanical hyperalgesia and thermal hyperalgesia, which are described in detail below.

3.2.3 Drug Preparation

For the persistent study, βAR antagonists ICI-118,511 (ICI; Tocris, Ellisville, MO) and SR59230A (SR; Tocris, Ellisville, MO) were dissolved together in a 5:3:2 ratio of DMSO, 0.9% saline, and ethanol. Drug solution or Vehicle (Veh) was injected into Alzet pumps, and pumps were connected to bifurcated catheters. Each pump/catheter system was placed into a 15mL conical tube containing sterile 0.9% saline and primed overnight in a dry heat bath

(Lab Armor, Cornelius, OR) at 37 degrees Celsius. Peripheral delivery of ICI was at a constant rate of 5.113mg/kg/day and peripheral delivery of SR was at a constant rate of 0.511mg/kg/day.

For the acute study, βAR antagonists ICI and SR were dissolved together in a 1:4 ratio of DMSO and 0.9% saline. A bolus dose of ICI (5.113mg/kg) and SR (0.511mg/kg) was provided via i.p. injection.

3.2.4 Surgical Procedures

Mice were anesthetized by isoflurane inhalation (4-5% induction, 1.5-3% maintenance). Hair was removed from incision sites by plucking, and incision sites were disinfected with ethanol and betadine. Sterile technique was employed throughout the duration of all procedures according to IACUC requirements. Stainless steel wound clips (Braintree Scientific, Braintree, MA) were used to close the wounds.

For i.pl. delivery of βAR antagonists, a modified version of the protocol published by Haddad et al[209] was used. Pumps were attached to a 7.4cm custom-made Y-shaped, bifurcated 3F-silicone catheter (SAI Infusion Technologies, Libertyville, IL). A small incision was made over the shoulder blades and hemostats were used to create a small s.c. pocket. The pump was implanted into the pocket, with the delivery port facing caudally. Small incisions were made over each hindlimb and a stainless steel 14G X 8.5cm semi-blunt trocar (SAI Infusion Technologies, IL) was used to route each catheter end from the incision at the shoulder blades to an incision made at either hindlimb. Each side of the bifurcated section of the catheter was sloped similarly towards its respective hindpaw, to ensure equal resistance on each side. The catheter ends were attached to fascia in the hindlimbs using 4-0 silk sutures (Oasis Medical, IL). Hemostats were then used to create small subcutaneous pockets in the hindlimbs, towards the hindpaws, creating resistance-free regions in which

each catheter end could be placed. Catheter ends were trimmed to fit into their respective subcutaneous pockets.

3.2.5 Assessment of Behavioral Responses to Mechanical and Thermal Stimuli

Mechanical allodynia was assessed using a 0.275g von Frey filament (Stoelting, Wood Dale, IL). This filament was chosen as a normally innocuous stimulus, as it has a gram force value under the withdraw threshold for normal wildtype mice. The filament was applied to the hind paw 10 times for a duration of 1 second each, with an interstimulus interval of 1 second.[147] The number of paw withdrawals (which could range from 0-10) was recorded for each hind paw at each time point. Mechanical allodynia was defined as an increase in the number of paw withdrawals in response to a normally innocuous mechanical stimulus. Mechanical hyperalgesia was tested similarly, but with a 4.000g von Frey filament. This filament was chosen as a normally noxious stimulus, as it has a gram force value well over the withdraw threshold for normal wildtype animals. Mechanical hyperalgesia was defined as an increase in the number of paw withdrawals in response to a normally noxious mechanical stimulus.

Paw withdrawal threshold was assessed using the von Frey up-down method.[212] Nine calibrated and logarithmically spaced von Frey monofilaments (bending forces: 0.005, 0.225, 0.275, 0.667, 1.627, 4.000, 6.824, 11.706, and 14.647g; Stoelting, Wood Dale, IL) were applied each plantar hind paw. First, the middle filament (1.627g) was applied to the hind paw for 1 second. If the mouse responded with a withdrawal, an incrementally lower filament was applied. In the absence of a withdrawal, an incrementally higher filament was applied. A series of 6 total responses were recorded for each paw. Results were entered into the Paw Flick module within the National Instruments LabVIEW 2.0 software (LabVIEW, Austin, TX), which uses a logarithmic algorithm to determine the gram force value that would elicit paw withdrawal in 50% of trials ($10^{(Xf+k\delta)}/10{,}000$, where X_f = value [in log units] of the

final von Frey hair used; k = tabular value of positive and negative responses, and δ = mean difference [in log units] between stimuli). Mechanical pain was defined as a decrease in paw withdrawal threshold.

Thermal hyperalgesia was assessed using the Hargreaves method.[213] Animals were placed in Plexiglass chambers and a radiant beam of light was applied to the hind paw through a glass floor. Paw withdrawal latencies were recorded in duplicate per paw. If the second latency recorded was not within ±4 seconds of the first, a third measure was recorded. The 2 latencies closest in value were averaged to determine overall latency to withdrawal. Thermal hyperalgesia was defined as a decrease in paw withdrawal latency in response to a noxious thermal stimulus.

3.2.6 Assessment of Non-Evoked Behavioral Responses

Weight was measured in grams using an animal weighing scale (Kent Scientific, Torrington, CT). Temperature was measured using a rectal thermoprobe (Physitemp, Clifton, NJ). Grip strength was measured using a calibrated dual sensor grip strength meter (Stoelting, Wood Dale, IL). Left and right paws were measured separately and summed together. Grip strength was measured 3 times at each time-point and the average was calculated to create an aggregate score.

3.2.7 Statistical Analyses

Sample sizes were selected based on their ability in previous, similarly structured rodent studies to accurately demonstrate behavioral differences between groups.[147,149,150] Mechanical allodynia, mechanical hyperalgesia, and thermal hyperalgesia data were analyzed by 2-way analysis of variance (ANOVA for Group X Time). In ANOVA analyses, Groups correspond to the separate groups on the graph of interest, as denoted by different symbols and names. Post-hoc comparisons were performed using the Bonferroni test, which

corrected for multiple comparisons. Statistical significance was defined as $P<0.05$. All statistical analyses were performed using GraphPad Prism (GraphPad Software, La Jolla, CA).

3.3 Results

3.3.1 Genetic COMT deficiencies produce mechanical and thermal pain

Genetic and pharmacologic alterations resulting in reduced COMT activity are associated with increased experimental pain and likelihood of developing persistent pain disorders. Both acute[147] and persistent[274] (chapter 2) administration of the COMT inhibitor OR486 results in enhanced mechanical and thermal pain in rats. To evaluate the effects of genetic COMT deficiencies on pain, responses to mechanical and thermal stimuli were measured in separate groups of COMT+/+, +/- and -/- mice over a 2-week period. Compared to COMT+/+, COMT-/- mice exhibited mechanical allodynia (group: $p<0.0001$; group x day: $p=0.4753$; Figure 3.1A) and mechanical hyperalgesia (group: $p<0.0001$; group x day: $p=0.9953$; Figure 3.1B); as well as decreased thresholds in response to mechanical (group: $p<0.0001$; group x day: $p=0.9777$; Figure 3.1C) and thermal (group: $p<0.0001$; group x day: $p=0.4248$; Figure 3.1D) stimuli throughout the duration of the experiment. COMT+/- mice did not exhibit enhanced pain as compared to +/+ mice. COMT+/+, +/- and -/- mice did not exhibit differences in weight (Figure 3.2A), temperature (Figure 3.2B) or grip strength (Figure 3.2C).

Sexual dimorphism was not observed, as both male and female COMT-/- mice developed mechanical allodynia (male group: $p<0.0001$, Figure 3.2A; female group: $p<0.0001$, Figure 3.2E) and mechanical hyperalgesia (male group, $p<0.0001$, Figure 3.2B; female group: $p<0.0001$, Figure 3.2F); and demonstrated decreased mechanical (male group: $p<0.0001$, Figure 3.2C; female group: $p<0.0001$, Figure 3.2G) and thermal (male

group: p<0.0001, Figure 3.2D; female group: p<0.0001, Figure 3.2H) thresholds. See Figure 3.2 for all sexual dimorphism data.

3.3.2 Peripheral βAR antagonist administration does not alter persistent COMT-dependent pain in COMT-/- mice

Next, the contribution of peripheral $\beta_{2/3}$ARs to mechanical and thermal pain was evaluated in separate groups of COMT+/+ and -/- mice receiving sustained i.pl. administration of ICI (5.113mg/kg/day) and SR59230A (0.551mg/kg/day) or vehicle over a two-week period (Figure 3.4). Peripherally delivered antagonist doses were selected based on their ability to block acute pharmacologic COMT-dependent pain behaviors in previous rodent studies.[147] As previous studies were conducted in rats, a prediction model considering interspecies pharmacodynamics and pharmacodynamics was used to determine the optimal dose for each antagonist in mice.[275] Similar to COMT-/- mice receiving vehicle, -/- mice receiving sustained i.pl. coadministration of the β_2AR antagonist ICI-118,511 and β_3AR antagonist SR59230A exhibited mechanical allodynia (group: p<0.0001; group x day: p=0.1442; Figure 3.4A) and hyperalgesia (group: p<0.0001; group x day: p=0.9976; Figure 3.4B); as well as decreased mechanical (group: p<0.0001; group x day: p=0.9756; Figure 3.4C) and thermal (group: p<0.0001; group x day: p=0.6512; Figure 3.4D) thresholds; as compared to +/+ mice.

To confirm that peripheral $\beta_{2/3}$ARs antagonists were unable to reverse COMT-dependent pain, we performed an acute study in which we injected COMT-/- mice with a bolus dose of ICI (5.113mg/kg) and SR (0.551mg/kg). Similar to the persistent study, COMT-/- mice receiving acute ICI-SR (i.p.) also exhibited mechanical allodynia (group: p<0.0001; group x time: p= 0.0584; Figure 3.5A) and hyperalgesia (group: p<0.0001, group x time: 0.2887; Figure 3.5B); and decreased mechanical (group: p<0.0001; group x time: 0.4686; Figure 3.5C) and thermal (group: p<0.0001; group x time: 0.9806; Figure 3.5D) thresholds; as compared to +/+ mice. See Figure 3.5 for all acute study data.

3.4 Discussion

IPDs are associated with abnormalities in adrenergic pathways, such that patients exhibit increased levels of catecholamines alongside diminished activity of COMT. Work by our group and others has shown that diminished activity of COMT is due, at least in part, to polymorphic variation in the corresponding COMT gene. To model the etiology of idiopathic pain in patients possessing the low activity COMT alleles, the present study utilized COMT-/- mice that express endogenously low levels of COMT throughout their lifespan. The results reveal that COMT -/- mice exhibit consistent levels of mechanical and thermal pain evaluated over a two-week period. Furthermore, β_2- and β_3AR antagonists known to block the development of persistent pain due to COMT pharmacologic inhibition are unable to reverse persistent pain due to COMT gene knockdown.

In the present study, we found that COMT-/- mice exhibit robust increases in response to mechanical and thermal stimuli that remain stable over time. This is in line with results from previous studies demonstrating that COMT-/- mice have increased thermal pain sensitivity and stress responses.[239,276,277] COMT-deficient mice also demonstrate increases in impulsive,[278] aggressive,[238] and fear[277,279] behaviors, impairments in cognitive performance[278] and emotional reactivity,[238] and abnormalities in immune cell expression.[280] Meanwhile, mice with overexpression of COMT have blunted thermal pain and stress responses.[239]

As previous preclinical and clinical studies have reported sex-specific differences in COMT-related phenotypes[238-242] and as males and females exhibit different COMT expression patterns,[243,244] we examined COMT-dependent pain in both sexes. Results demonstrated that both male and female COMT-/- mice exhibit comparable increases in pain behaviors as compared to wildtype mice. This is consistent with results from Chapter 2, which demonstrated no sex-specific differences for pharmacologically induced COMT-dependent pain behaviors in rats.[274] However, this study was not specifically designed nor

statistically powered to find such effects, if indeed they exist, and future studies and clinical applications related to COMT-dependent pain should continue to consider possible sex-specific effects.

The present study also examined a non-evoked pain behavior, grip strength, in COMT-/- mice in attempt to capture spontaneous, unprovoked pain. Grip strength has been used in previous literature as a measure of non-evoked pain or discomfort[281,282] and we therefore hypothesized that COMT-/- mice would have decreased grip strength in comparison to wildtype mice. Interestingly, no difference in grip strength was found between genotypes. This may suggest that COMT-/- mice do not experience spontaneous pain. In contrast, this behavioral assay may not be relevant for an animal model of idiopathic pain. Limitations to the grip strength assay are that it may be more of a measure of function or motor skills than of pain,[283-287] and that it may be a more effective marker of inflammatory or joint pain, rather than idiopathic pain that results from genetic variance.[281,282,288] Future studies should utilize non-evoked pain assays that might be more relevant to idiopathic pain, such as the grimace scale.[289]

As β_2- and β_3ARs are known to mediate the initiation of COMT-dependent pain, we next examined the ability of coadministration of β_2- and β_3AR antagonists to block the maintenance of pain in COMT-/- mice. We found that coadministration of β_2- and β_3AR antagonists is unable to reverse mechanical and thermal pain observed in COMT-/- mice. This contradicts earlier rat studies that have demonstrated a role for β_2- and β_3ARs in the initiation of acute[147,149] and persistent[274] COMT-dependent pain. The discrepant findings may be due to 1) a distinct role for βARs in the development versus maintenance of COMT-dependent pain, 2) the duration of COMT inhibition, and/or 3) compensatory effects that sometimes accompany genetic manipulation.

The inability of β_2- and β_3AR antagonists to reverse pain in COMT-/- mice may be because they are more important for the development of pain than they are for the

maintenance of pain. In the pharmacologic rat model, peripheral β_2- and β_3AR antagonists were provided alongside the COMT inhibitor. Though this demonstrates their efficacy in blocking the development of COMT-dependent pain, it does not provide evidence that βAR antagonist treatment can reverse pain. Future studies should therefore examine the efficacy of peripheral β_2- and β_3AR antagonist treatment in the pharmacologic rat model, after COMT-dependent pain has already been established.

Another explanation for the negative effect of βAR antagonists on pain in COMT-/- mice could be because COMT-/- mice may possess developmental abnormalities. Catecholamines and catecholamine enzymes are present at early developmental stages.[290] The importance of catecholamine and βAR signaling in embryonic development has been demonstrated in various animal models.[291,292] In both animals and humans, levels of catecholamines can be represented by a "U" curve. Moderate levels of catecholamines are ideal, whereas very high or low levels are associated with deficits in neural circuitry, physiological reactivity, and executive function, particularly in response to stress.[293] In COMT-/- mice and patients with genetic COMT-dependent pain, increased catecholamine levels may lead to physiological changes beginning during early development. These early developmental changes would not be present in the pharmacologic rat model of COMT-dependent pain or in non-genetic COMT-dependent clinical pain. This could explain why COMT-/- mice are unresponsive to βAR antagonist treatment. Future studies should assess the efficacy of βAR antagonists in inducible COMT-/- mice to determine if βAR antagonists block pain in COMT-/- mice that have developed normally as COMT+/+ mice.

Finally, COMT-/- mice have more dramatic and continuous decreases in COMT than the pharmacologic rat model. As a result, ongoing pain signaling and overstimulation of βARs in COMT-/- mice may cause long-term impairments to various pathways and systems. For example, we know that COMT-dependent pain is accompanied by increases in inflammatory mediators such as nitric oxide (NO), interleukin 6 (IL-6), and tumor necrosis

factor-α (TNFα).[149] Circulating inflammatory mediators can result in the release of various growth factors (*e.g.,* granulocyte-macrophage colony-stimulating factor, nerve growth factor) and neuropeptides (*e.g.,* substance P, histamine, calcitonin gene-related peptide) that are involved with the maintenance of hyperalgesia.[294] This "inflammatory soup" can activate various other downstream pathways that promote pain, such as the mitogen-activated protein kinase (MAPK) pathway, and can ultimately result in transcription of nociceptive molecules and the hyper-excitability of nociceptive fibers.[295,296] Long-term impairments in signaling of these and other pathways may cause βAR antagonist administration to be ineffective for treatment of pain in COMT-/- mice. These same impairments are likely not present in the pharmacologic model, in which continuous βAR signaling is not present and COMT deficiencies are transient. Future studies should aim to identify downstream pathways affected by COMT abnormalities.

MicroRNAs may be helpful in identifying affected pathways downstream of COMT-dependent pain in future studies. MicroRNAs (miRNAs) are small, non-coding pieces of RNA that can regulate gene expression by binding to downstream mRNA transcripts.[297] MiRNA dysregulation has been implicated in several diseases, including disorders related to pain and inflammation.[93,298] MiRNAs also play a role in catecholamine and βAR signaling.[181-184] Identifying miRNA expression profiles for the pharmacologic rat model of COMT-dependent pain, the genetic mouse model of COMT-dependent pain, and clinical pain conditions may help us to identify pathways that are affected for different types and stages of persistent COMT-dependent pain. Successful treatment of COMT-dependent pain may require targeting βARs in addition to pathways affected by genetic and/or environmental COMT abnormalities. Thus, the study of miRNA expression profiles as they relate to COMT-dependent pain may help to identify better therapeutic options.

In conclusion, we utilized a clinically relevant, genetic animal model of COMT-dependent pain to demonstrate that, though the initiation of COMT-dependent pain is

mediated *via* peripherally located β_2- and β_3ARs, the maintenance is not. It remains possible that βAR antagonist treatment may be most effective for preventing the development of non-genetic COMT-dependent pain, particularly following an environmental event such as a car accident or surgery. Though βAR antagonist treatment may be beneficial to a subset of patients, alternative multi-target treatment plans may be necessary for those with genetic COMT-dependent pain, who may have substantial downstream abnormalities as a result of early developmental problems or persistent pain signaling. Future work in this area may help to stratify COMT-dependent patients into treatment subgroups, as well as to inform clinicians on how to most effectively utilize βAR antagonists for the treatment of persistent pain.

3.5 Acknowledgements

The authors thank SAI-Infusion Technologies for their assistance with catheter design.

3.6 Footnotes

[1] This work was funded by R01 NS072205 to A.N and P01 NS045685 to A.N (NIH/NINDS, Bethesda, MD, USA 20892).

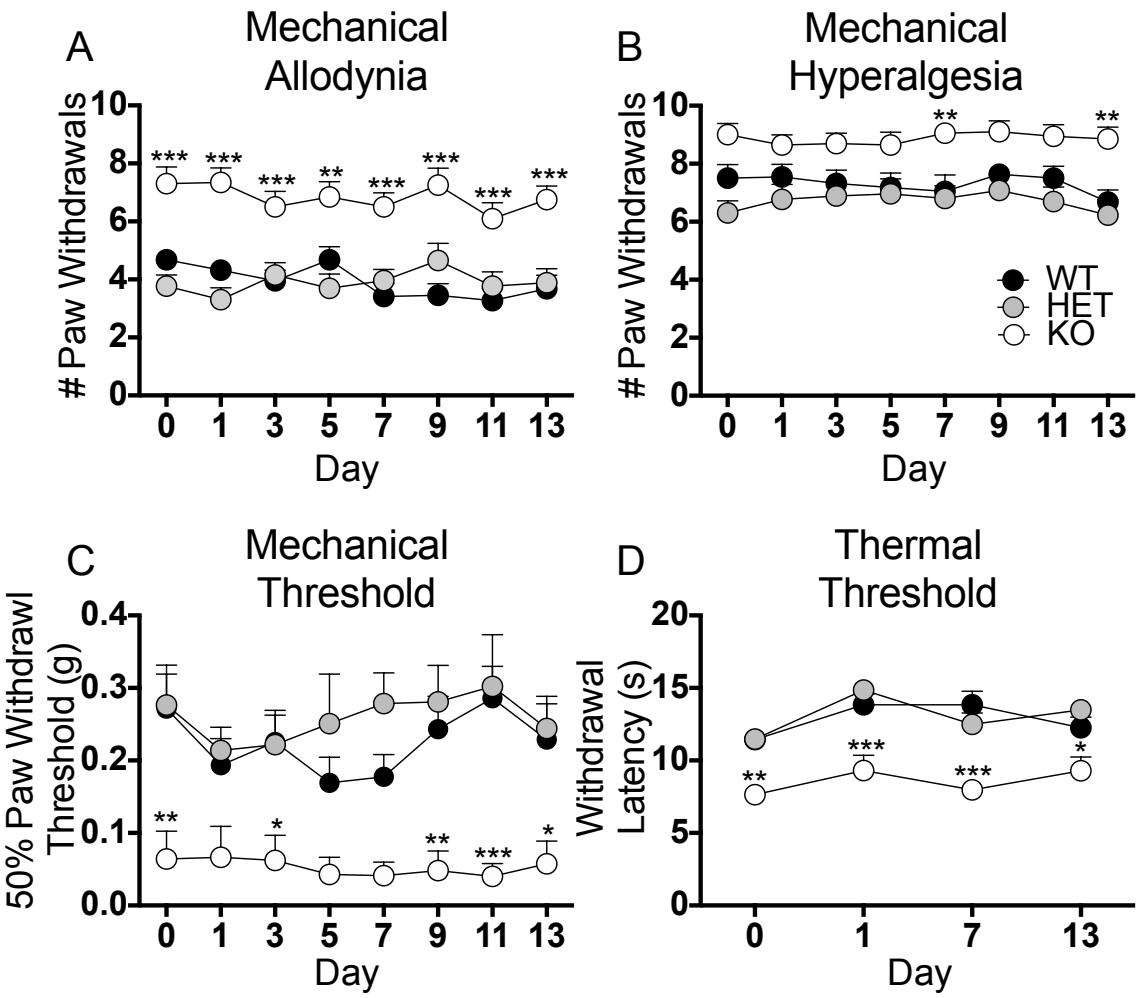

Figure 3.1 COMT-/-, but not COMT +/-, mice have enhanced mechanical and thermal pain. Compared to COMT +/+ and +/- mice, -/- mice demonstrate an increase in **(A)** mechanical allodynia and **(B)** hyperalgesia; and a decrease in **(C)** mechanical and **(D)** thermal thresholds. N=10-13 per group (5-7F and 4-6M). Data are expressed as mean ± SEM. ***P<0.001, **P<0.01, *P<0.05 different from WT. Abbreviations: catechol-O-methyltransferase (COMT), female (F), grams (g), heterozygote (HET, +/-), knockout (KO, -/-), male (M), seconds (s), wildtype (WT, +/+).

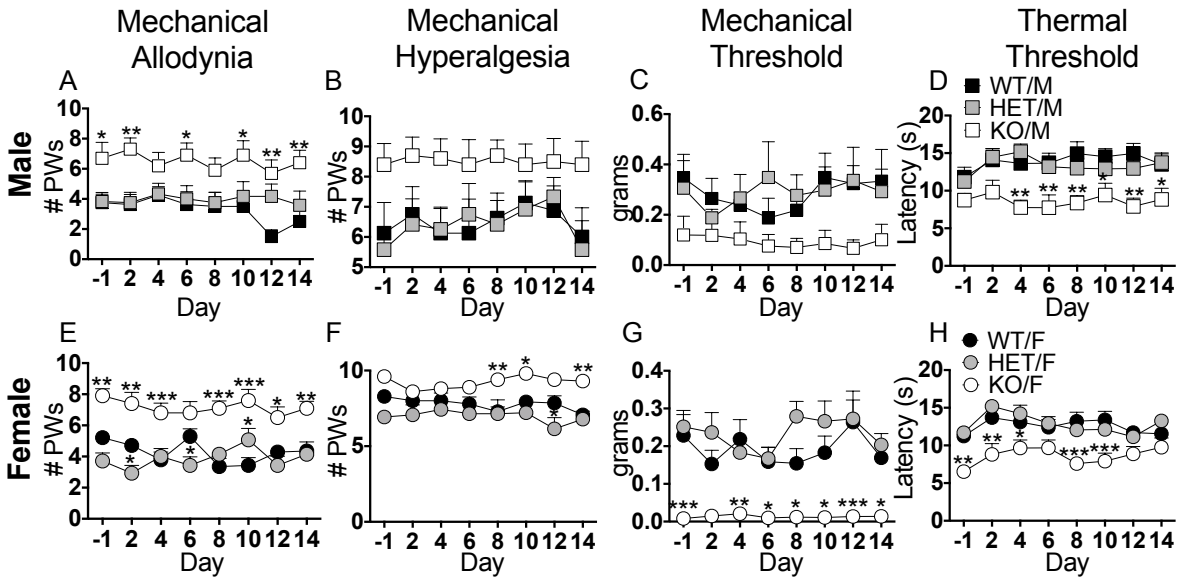

Figure 3.2 Enhanced mechanical and thermal pain sensitivity occurs in both male and female COMT -/- mice. Compared to COMT +/+ and +/- mice, -/- mice demonstrate an increase in **(A,E)** mechanical allodynia and **(B,F)** hyperalgesia; and a decrease in **(C,G)** mechanical and **(D,H)** thermal thresholds; in both males and females. N=5-7 per group. Data are expressed as mean ± SEM. ***P<0.001, **P<0.01, *P<0.05 different from WT. Abbreviations: catechol-O-methyltransferase (COMT), female (F), heterozygote (HET, +/-), knockout (KO, -/-), male (M), paw withdrawal (PW), seconds (s), wildtype (WT, +/+).

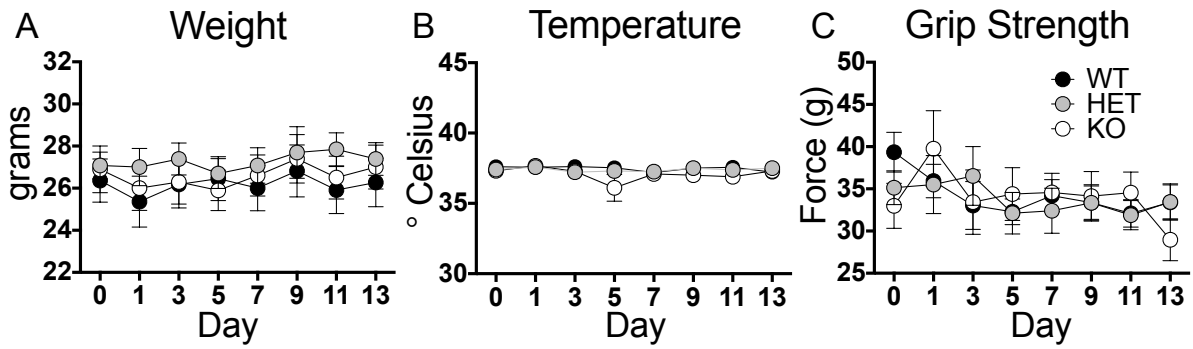

Figure 3.3 COMT +/+, +/-, and -/- mice display no differences in weight, temperature, or grip strength. Compared to COMT +/+ and +/- mice, -/- mice demonstrate no difference in **(A)** weight, **(B)** temperature, or **(C)** grip strength. N=10-13 per group (5-7F and 4-6M). Data are expressed as mean ± SEM. Abbreviations: catechol-O-methyltransferase (COMT), female (F), grams (g), heterozygote (HET, +/-), knockout (KO, -/-), male (M), wildtype (WT, +/+).

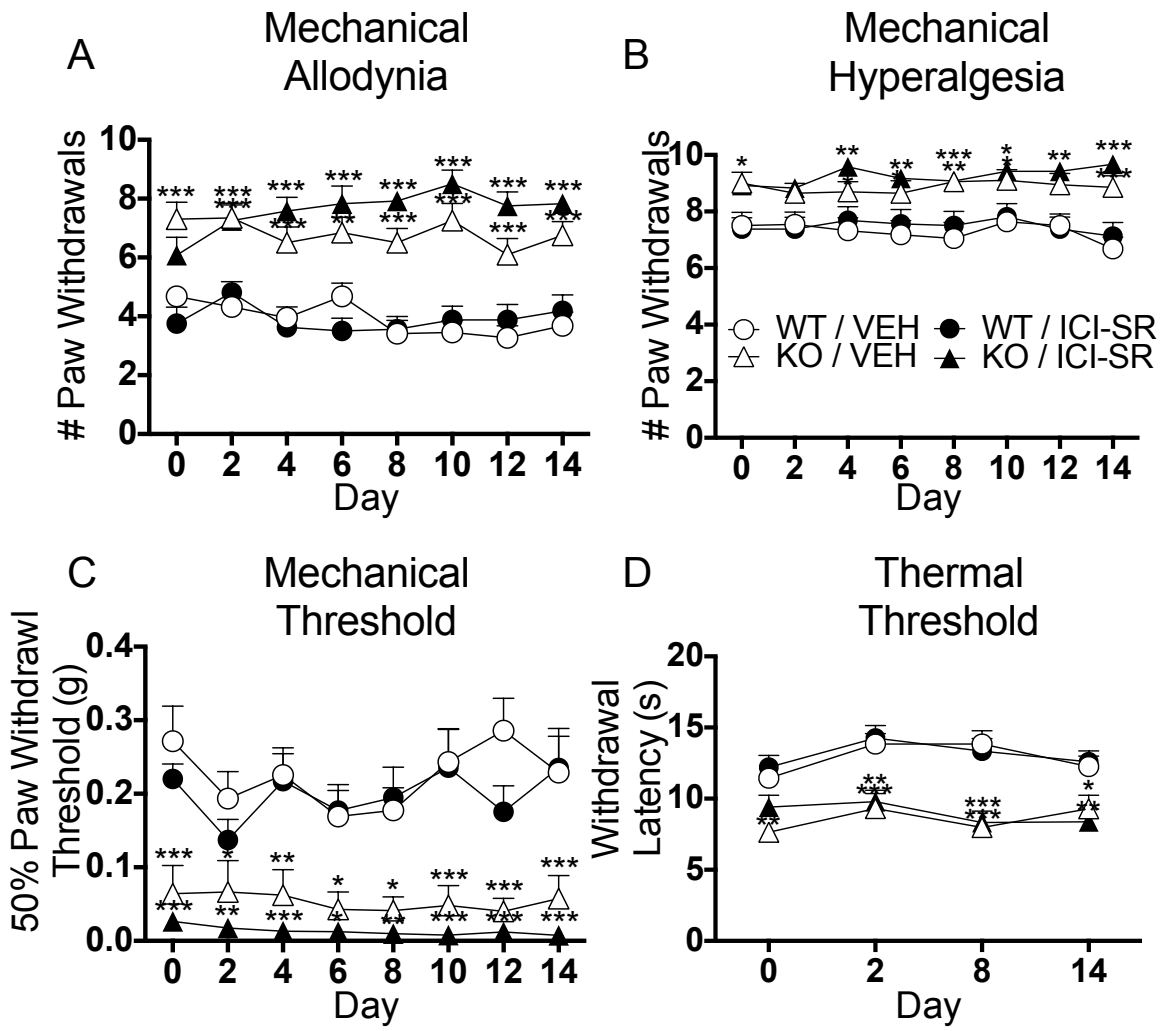

Figure 3.4 Sustained peripheral administration of $\beta_{2/3}$AR antagonists does not alter COMT-dependent pain in COMT-/- mice. Peripheral coadministration of the β_2AR antagonist ICI-118,551 and the β_3AR antagonist SR59230A fails to reduce **(A)** mechanical allodynia and **(B)** hyperalgesia or increase **(C)** mechanical or **(D)** thermal threshold. N=6-11 per group (5-7F and 1-5M). Data are expressed as mean ± SEM. ***P<0.001, **P<0.01, *P<0.05 different from WT/Veh. Abbreviations: female (F), grams (g), heterozygote (HET, +/-), ICI-118,551 (ICI), knockout (KO, -/-), male (M), seconds (s), SR59230A (SR), Vehicle (Veh), wildtype (WT, +/+).

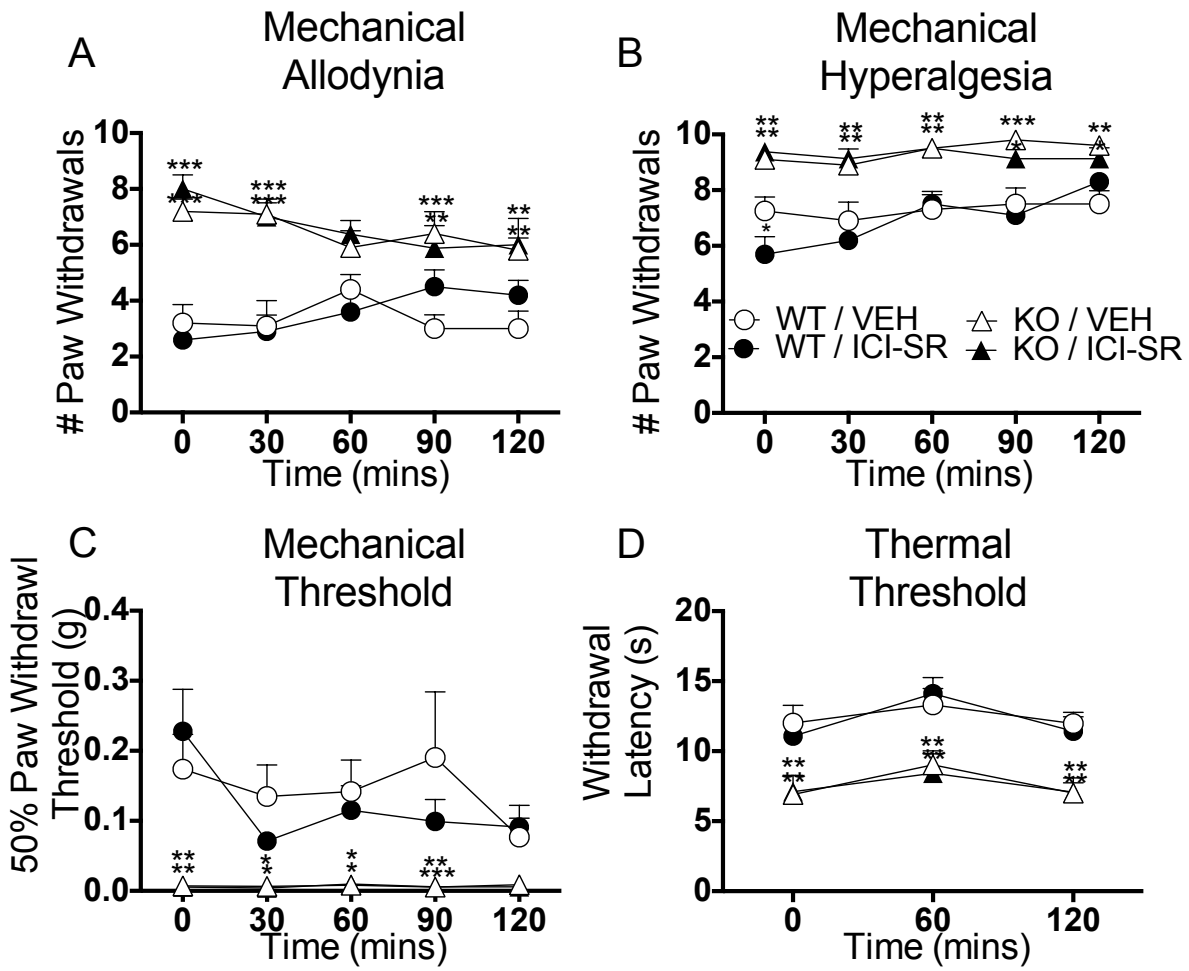

Figure 3.5 Acute administration of $\beta_{2/3}$AR antagonists does not alter COMT-dependent pain in COMT-/- mice. Acute i.p. delivery of the β_2AR antagonist ICI-118,551 and the β_3AR antagonist SR59230A in a bolus dose does not block **(A)** mechanical allodynia or **(B)** hyperalgesia; nor does it affect **(C)** mechanical or **(D)** thermal threshold of COMT-/- mice. N=4-5 per group (2F and 2-3M). Data are expressed as mean ± SEM. ***P<0.001, **P<0.01, *P<0.05 different from WT/Veh. Abbreviations: female (F), grams (g), heterozygote (HET, +/-), ICI-118,551 (ICI), intraperitoneal (i.p.), knockout (KO, -/-), male (M), seconds (s), SR59230A (SR), Vehicle (Veh), wildtype (WT, +/+).

CHAPTER 4

MICRORNA EXPRESSION PROFILES DIFFERENTIATE IDIOPATHIC PAIN DISORDER SUBTYPES [1,2]

4.1 Introduction

Vestibulodynia (VBD) is an idiopathic pain disorder (IPD), characterized by entry dyspareunia, tenderness to touch and presence of erythema on the vulvar vestibule, that affects up to 15% of women in the United States.[299,300] VBD often co-occurs with other IPDs including irritable bowel syndrome (IBS; 35%)[301], temporomandibular disorder (TMD; 78%),[299] and fibromyalgia (FMS; 17%).[301] VBD and related IPDs represent a significant healthcare problem, together affecting up to one billion adults worldwide.[302] Current treatment regimens remain ineffective due to the conditions' uncertain etiology and heterogeneous clinical manifestation.[299] To understand the nature of these complex conditions and improve standards of care, the identification of unique biological signatures and pathways that map onto distinguishing clinical features is required.

While clinical manifestations are heterogeneous, VBD and related IPDs are associated with a state of pain amplification, psychological distress and enhanced inflammation.[91] In VBD, pain is localized to the pelvis, possibly due to altered permeability or cellular composition of the mucosa.[299] Women with VBD demonstrate higher levels of anxiety and somatization as well as enhanced production of pro-inflammatory cytokines.[300] Compared to individuals with one IPD, those with co-occurring IPDs exhibit increased psychological distress[299] and imbalances in pro- and anti-inflammatory mediators possibly indicative of abnormalities in central pain processing.[32] MicroRNAs (miRNAs) represent biological determinants of pain, mood, and inflammation. miRNAs are small, noncoding

pieces of RNA that control gene expression by inhibiting protein translation or degrading downstream target mRNAs.[297] Aberrant miRNA profiles have been associated with several animal models of pain and inflammation as well as painful conditions in humans.[93] Further, miRNA profiling represents a novel and clinically-relevant approach for patient stratification of pain-related conditions.[121] Emerging evidence also suggests a role for miRNAs in psychological conditions such as depression and anxiety.[303] Lastly, miRNAs regulate genes involved in activation of immune cells and secretion of inflammatory cytokines.[104] This suggests that miRNAs are key contributors to the pain amplification, psychological distress and enhanced inflammation characteristic of IPDs such as VBD.

Here, we aimed to elucidate novel clinical features and biological pathways unique to women with VBD and to those with VBD plus a commonly co-occurring IPD, IBS (VBD+IBS). The primary aim was to identify miRNAs that differ in expression between groups. Secondary aims were to describe differences among clinical groups in pain-related phenotypes signifying clinical, psychological, pain-relevant, and inflammatory characteristics. We found that women with VBD have pain localized to the pelvis, normal self-reported pain and psychological profiles, increased levels of pro- and anti-inflammatory cytokines, and dysregulation of miRNAs predicted to be involved in pain processing and estrogen signaling. Women with VBD+IBS have greater pain sensitivity at remote bodily sites, enhanced self-reported pain and somatization, imbalanced pro- and anti-inflammatory responses, and dysregulation of miRNAs predicted to be involved in pain processing, cellular physiology, and central sensory pathways. Collectively, these results suggest miRNA profiles may be useful for understanding the shared and unique mechanisms of localized versus widespread pain conditions.

4.2 Materials and Methods

4.2.1 Subject Consent and Enrollment

All subjects were enrolled after giving informed consent as approved by the Biomedical Institutional Review Board of the University of North Carolina at Chapel Hill (UNC).

4.2.2 Inclusion and Exclusion Criteria

This study utilized data from 78 women (33 VBD, 23 VBD+IBS, and 22 HC). Subjects in VBD and VBD+IBS groups were recruited at the UNC Pelvic Pain Clinic and subjects in the HC group were recruited through fliers placed on campus and in the local community between August 2008 and August 2010. Sample sizes were based upon prior miRNA studies.[115,121,304,305] Preliminary eligibility assessment of interested subjects was conducted *via* a phone interview. Subjects were excluded for a positive response to any of the following criteria: 1) age <21 or >45; 2) breastfeeding, pregnant, or menopausal; 3) significant medical conditions such as seizure disorder, diabetes, or thyroid disorder; and 4) known diagnosis of comorbid urogenital pain conditions such as interstitial cystitis. Subjects without vulvovaginal complaints were recruited as potential controls.

Subjects were then clinically assessed during a standardized gynecological exam during which they rated pain using the modified Gracely Pain Scale.[306] VBD was diagnosed in women who reported a history of pain during intercourse or tampon insertion and who experienced tenderness to touch upon palpation of the vestibule with a cotton swab during the exam.[307] The threshold for pain history was a rating of 3 or more using the Gracely Pain Unpleasantness Scale. During the clinical exam, subjects with suspected dermatological disorders (*e.g.,* lichen sclerosis, contact dermatitis) and vaginismus were excluded. All exams were performed by a single examiner.

Subjects were also assessed for other pain disorders including IBS, FM, TMD, and migraine headaches. IBS case status was determined using 4 abdominal pain questions in

accordance with Rome III Criteria.[308] Multicenter Criteria were used to classify FM, defined by widespread pain in combination with tenderness at 11 or more of the 18 specific tender point sites.[309] Classification of TMD was based on the Research Diagnostic Criteria for temporomandibular disorders, with the defining characteristics being a history of facial pain and examiner-evoked pain reported in the cheeks, jaw muscles, temples, or jaw joints.[310] Migraine headaches (with or without aura) were classified by the International Classification of Headache Disorders (ICHD-2) criteria.[311] As the majority of the VBD subjects with co-occurring pain syndromes had IBS, the following 3 groups were created: subjects with VBD alone, subjects with VBD and IBS (including those with or without additional co-occurring pain disorders), and pain-free healthy controls. Healthy controls were excluded if they reported any psychological or pain-related conditions.

4.2.3 Mucosal Pressure Pain Measurement

Twelve pressure points of the vestibule were defined with reference to a clinical drawing annotated with a conventional clock face. The 12 o'clock position marked the anterior midline and the 6 o'clock position marked the posterior (in dorsal lithotomy position). Six sites were assessed for pain: 3 on the upper vestibule at positions 2, 10, and 12; and 3 on the lower vestibule at points 5, 7, and 6, in that order.[306] To account for subject-to-subject variability, anatomical landmarks were also used to standardize the location of vestibular sites between women. For each pelvic mucosal pain assay averaging the 6 site scores together created an aggregate score.

Intensity: To assess pain sensitivity in the vestibule the examiner applied pressure to each site with a Q-tip and instructed the subject to rate her pain on a scale of 0-10 (0=no pain, 10=worst imaginable). For mucosal intensity assays: HC N=21, VBD N=33, and VBD+IBS N=23.

Threshold: A digital vestibular pressure algometer designed by the Center for Pain Research and Innovation at UNC and first described by Zolnoun et al[306] was used to record the threshold to pressure pain sensitivity in the vestibule. The algometer is attached to a cotton swab that was applied to each site beginning at 1N and increasing until the subject's first sensation of pain, at which time the subject was instructed to click a computer mouse. The latency was recorded in real-time. This test was repeated on each site 3 times with an interstimulus interval of 2 seconds. Results report the average score of the 3 tests. For mucosal threshold assays: HC N=13, VBD N=31, and VBD+IBS N=20.

4.2.4 Muscle Pressure Pain Detection Measurement

To test muscle pressure pain of the vulvovaginal muscles, a pressure sensor was affixed to a plastic thimble that was worn over the investigator's right index finger. The algometer pressure sensor, which measured forces ranging from <1N to >98N, was used for direct and isolated palpation of the right and left puborectalis levator muscles (sites 5 and 7) and perineal muscle complex (site 6). An aggregate score was calculated as the average of the 3 muscular sites. The exam to test for muscle pressure pain was conducted transvaginally in accordance with conventional clinical practice.[306]

Intensity: Examiner applied pressure to each site on the lower vestibule and had the subject rate her pain (0=pressure, 1=low, 2=moderate, and 3=severe). For muscle intensity tests: HC N=21, VBD N=33, and VBD+IBS N=23.

Threshold: Subjects were instructed to click a computer mouse at the first sensation of pain to find the muscle pain perception threshold, which was recorded in real-time by the computer. The examiner terminated the pressure if she was unable to apply additional force to reach the subject's threshold. For muscle threshold tests: HC N=16, VBD N=30, and VBD+IBS N=19.

Tolerance: The subject was then given the option to undergo tolerance testing, which was performed after a 5-minute rest period. 61/78, or 78% of subjects opted to participate. Subjects were instructed to click the computer mouse when they were no longer able to tolerate the pressure. For muscle tolerance tests: HC N=15, VBD N=28, and VBD+IBS N=18.

4.2.5 Remote Bodily Pressure Pain Threshold Measurement

A digital algometer (Wagner, Greenwich, CT, USA) was applied for 3 trials to each side of the trapezius and the temporomandibular joint (TMJ) beginning at a pressure of 1 N and increasing until the subject's first sensation of pain, at which time the threshold force was recorded in Newtons. Final scores report the average of left and right thresholds for each site. For remote bodily pressure pain measurements: HC N=23, VBD N=30, and VBD+IBS N=20.

4.2.6 Remote Bodily Thermal Windup Measurement

Thermal stimuli of 50°C were applied repeatedly to the right hand to evaluate the temporal summation of thermal heat pain "windup".[312] Ratings of pain in response to 10 repeated stimuli were evaluated. Stimuli of 0.5 sec duration were repeated once every three seconds and pain was rated on a 0–100 numerical rating scale (NRS). The procedure was stopped if the participant reported a rating of 100 or if she asked that it be stopped. For thermal windup measurements: HC N=21, VBD N=30, and VBD+IBS N=19.

4.2.7 Assessment of Psychological and Self-reported Health Phenotypes

Prior to clinical examination, subjects completed questionnaires that collectively assessed affective components of pain, somatic symptoms related to pain, perceived control, self-rated health and mood. The McGill Pain Questionnaire (MPQ) assessed sensory components of pain using 11 verbal describers and affective qualities related to pain using 5 describers. Responses on 4-point scales were summed to compute scores for

each section.[32] The Short Form 12 version 2 (SF12v2) assessed 6 domains: global health, physical functioning, physical roles, emotional functioning, emotional roles, and pain interference; using an algorithm[313] based on answers to 12 physical and mental health related questions. Each scale ranged from 0 to 100, with higher values signifying better function and mood.[32] Answers to 54 questions on the Pennebaker Index of Limbic Languidness (PILL) questionnaire were used to create a summary score of somatic symptoms (*e.g.,* itchy eyes, dizziness). Frequency for each symptom was recorded on a five-point Likert scale ranging from "never or almost never" to "more than once a week".[32] The Comprehensive Pain and Symptom Questionnaire (CPSQ) assessed various components and effects of bodily pain (*e.g.,* face, jaw, head, and lower back pain) using individual scaled scores from answers to 55 questions.[14] The Symptom Checklist 90-Revised (SCL-90R) used 90 questions in to assess a broad range of psychological symptoms such as anxiety and depression.[314]

4.2.8 Assessment of circulating cytokine protein levels

During the clinic visit, whole blood was collected into ethylenediaminetetraacetic acid-coated Vacutainer tubes (BD Biosciences, Franklin Lakes, NJ, USA) and placed on ice. Plasma was isolated by centrifuging at 1520 RPM for 10 minutes at 4°C and aliquoted into Cryotubes (Nalge Nunc International, Lima, OH, USA), frozen with liquid nitrogen and stored at -80°C. Samples were thawed on ice and the Fluorokine MAP Multiplex Human Cytokine Panel A (R&D Systems, Minneapolis, MN, USA) was used to measure the levels of 22 cytokines, including monocyte chemotactic protein-1 (MCP-1), macrophage inflammatory protein-1α (MIP-1α), macrophage inflammatory protein-1β (MIP-1β), regulated upon activation normal t-cell expressed and secreted (RANTES), epithelial-derived neutrophil-activating peptide 78 (ENA-78), fibroblast growth factor basic (FGF basic), granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-

CSF), interferon-γ (IFN-γ), interleukin-1α (IL-1α), interleukin-1β (IL-1β), interleukin-1 receptor antagonist (IL-1ra), interleukin-2 (IL-2), interleukin-4 (IL-4), interleukin-5 (IL-5), interleukin-6 (IL-6), interleukin-8 (IL-8), interleukin-10 (IL-10), interleukin-17 (IL-17), tumor necrosis factor α (TNFα), thrombopoietin (Tpo), and vascular endothelial growth factor (VEGF). A full list of cytokines including detection thresholds can be found here: http://www.rndsystems.com/product_detail_objectname_fmaphumansample.aspx.

Plasma samples were incubated first with a set of color-coded beads pre-coated with antibodies for each of the 22 cytokines and then with corresponding biotinylated secondary antibodies. Samples were then incubated with streptavidin-phycoerythrin conjugate and read with a Luminex dual laser analyzer (Luminex Corporation, Austin, TX, USA), which detects the magnitude of the phycoerythrin signal to determine cytokine levels. Standards were measured in duplicate and then the mean fluorescent intensity was calculated for each sample. The experimenter was blinded to experimental groups for all cytokine assays.

4.2.9 miRNA Profiling

During the clinical visit, whole blood was also collected in PAXgene Blood RNA tubes (BD Biosciences, Franklin Lakes, NJ, USA) and stored at -80°C. After thawing tubes, RNA was isolated from blood using PAXgene blood miRNA kits (Qiagen, Germantown, MD, USA). RNA concentrations were measured on a NanoDrop 1000 (NanoDrop Technologies, Wilmington, DE, USA) and normalized to 20ng/uL using TE buffer. cDNA synthesis was performed using the TaqMan Reverse Transcription Kit, along with human Pool A and B Megaplex RT primers. cDNA was preamplified using TaqMan Preamplification Master Mix and human primers that corresponded to the Pool A and B RT primers. Samples were prepared and loaded onto OpenArray plates by the AccuFill Robot System (Life Technologies, Grand Island, NY, USA) using the protocol recommended by the vendor. Loaded OpenArray plates were run through the QuantStudio 12K Flex Real-Time

OpenArray PCR System (Life Technologies, Grand Island, NY, USA) to assess expression of 761 commonly-studied miRNAs. RT-PCR was used to verify results for 5 miRNAs of interest using the Taqman Universal PCS Master Mix, Taqman miRNA Assays (Life Technologies, Grand Island, NY, USA) and the protocol recommended by the vendor. The experimenter was blinded to experimental groups for all miRNA assays.

4.2.10 miRNA Pathway Analysis

MiRNA expression profiles in blood may inform us of both peripheral and central pain processes affected in subtypes of IPDs. Samples of circulating blood provide a rich source of pain-relevant molecules, including neurochemicals released by sympathetic nerve terminals and inflammatory mediators released by circulating immune cells.[315] Furthermore, studies have shown that whole blood shares significant gene[316] and miRNA[317] expression similarities with CNS tissues.

To determine the genes and pathways affected by miRNA expression in women with VBD and VBD+IBS, we performed pathway analysis using the *in silico* Multiple MicroRNA Analysis Software provided by the Diana Lab (http://diana.cslab.ece.ntua.gr/pathways/index_multiple.php). This software program identifies predicted and experimentally validated gene targets of miRNAs using an advanced algorithm that is based on levels of interactions between miRNAs and their target genes. The program next organizes these genes of interest into Kyoto Encyclopedia of Genes and Genomes (Kegg) pathways. For each pathway, a union –ln(p-value) that accounts for all genes regulated by miRNAs of interest was also generated.

4.2.11 Statistical Analysis

Summary scores from questionnaires and clinical assessments were compared among cases and controls using 2-tailed t-tests with the Benjamini-Hochberg procedure for multiple testing corrections. For all mucosal, muscle, and remote bodily pressure pain

measurements, groups were compared using one-way ANOVA and the Dunnett correction method. The thermal windup data were analyzed using a linear mixed model for repeated measures. The participant dependent variable was the numeric rating of pain in response to each stimulus. Fixed effects were clinical case classification (3 groups, modeled as a categorical variable), the stimulus sequence (continuous variable, 1-10), and the square of the stimulus sequence (continuous variable, 1-100). Interaction terms between case-classification and each of the continuous variables were included to test for differences in the rate of windup among clinical sub-groups. The random effect was person (categorical variable) and a variance components covariance structure was specified for the random effects. Cytokine expression levels were compared among groups using one-way ANOVA and the Dunnett correction method. All comparison analyses were completed using GraphPad Prism (Prism, La Jolla, CA, USA).

4.2.12 miRNA Data Analysis

Raw data of miRNA Cycle Threshold (CT) were filtered by at least 3 expressed (*i.e.,* CT<32) samples within either HC group or combined case group. This filtration process permitted assessment of miRNAs that were present in all groups, as well as those that were largely increased or decreased in either case or control groups. Of the 761 human miRNAs, 250 were excluded, leaving 511 miRNAs for analysis. The filtered data were imported into DataAssist (Life Technologies, Grand Island, NY, USA) to calculate fold changes versus controls using CT values and the 2^{-CT} method. Data were normalized by the Global Normalization method[318] using median CTs with maximum allowable CT of 32 and including max CT in the calculations option, then 2^{-CT} values were exported from DataAssist software as relative expression values. Secondary normalization using Variance Stabilization method[319] were performed with the vsn package in Bioconductor (http://www.bioconductor.org/) and the output is log2 transformed normalized relative

expression values. Differential expression of miRNAs between case and control groups was tested by ANOVA or ANCOVA models using Partek GenomicSuite software (Partek, St. Louis, MO, USA). Including run and plate as fixed effect factors in the models controlled for batch effect between different runs and assay plates. An ANCOVA model was used to remove the effect of age as a covariate on the results. miRNAs of interest from the differential expression analyses were picked based on combination of p-value and fold change filters indicated in the report. Linear regression analyses between miRNAs and intermediate phenotypes were performed in Partek, with correlation p-values adjusted for multiple testing using the Benjamini-Hochberg (step-up) false discovery rate. Reported correlations are with $r<-0.40$ or $r>0.40$, and adjusted $p<0.05$.

4.3 Results

4.3.1 Presence of Comorbid Pain Conditions:

Data were collected from 33 women with VBD, 23 with VBD+IBS, and 22 HC. Of those with VBD+IBS, 8 displayed additional pain conditions: TMD (n=4) or TMD and FM (n=4) Subjects were demographically similar (Table 4.1).

4.3.2 Pelvic Pressure Pain Associated with Case Status:

Pain evoked in response to pressure applied to the pelvic muscles and mucosa is a primary symptom of VBD. Average responses to pelvic muscle tests were similar at each site (Figure 4.2) therefore data were collapsed across all 3 muscle sites. Compared to HC, women with VBD and VBD+IBS had increased stimulus-evoked muscle pressure pain. Women with VBD+IBS reported the greatest evoked muscle pain intensity ratings ($F_{2,228}=19.74$, $p<0.0001$; Figure 4.1A) and the lowest muscle threshold scores ($F_{2,191}=10.02$, $p<0.0001$; Figure 4.1B). No differences in muscle pain tolerance were observed (Figure

4.2G-I). Average verbal intensity and threshold responses to pelvic mucosal stimulation were similar at each site (Figure 4.3); therefore data were collapsed across all 6 mucosal sites. Compared to HC, women with VBD and VBD+IBS reported enhanced pain intensity in response to pressure applied to the pelvic mucosa ($F_{2,459}$ =37.26, p<0.0001; Figure 4.1C). Women with VBD and VBD+IBS did not, however, exhibit reductions in pelvic mucosal pain thresholds (Figure 4.1D) as compared to HC. These data suggest that muscular pain, which is characterized by increased pain intensity and decreased thresholds, is a primary feature of VBD and VBD+IBS. The magnitude of pain evoked by mucosal stimulation is less pronounced in VBD and VBD+IBS compared to pain evoked by muscle stimulation.

4.3.3 Remote Bodily Pain Associated with Case Status:

Pain at remote bodily sites was evaluated to determine if pain was generalized to other body regions. When a pressure stimulus was applied to the trapezius (Figure 4.1E) or the TMJ (Figure 4.1F), women with VBD+IBS exhibited a tendency towards reduced pain thresholds compared to HC or women with VBD alone. Similar group differences were observed in response to thermal heat repeatedly applied to the forearm. The 1st stimulus elicited similar ratings for all groups. Successive stimuli then elicited greater pain among VBD+IBS subjects compared to controls whose pain ratings plateaued after the 6th pulse, indicating that women with VBD+IBS have a greater capacity to temporarily summate heat pain. Intermediate effects were observed for the VBD group (Figure 4.1G). In sum, these data suggest that women with VBD+IBS experience enhanced pain in response to pressure and thermal stimuli at remote bodily regions, indicative of central sensitization.

4.3.4 Self-reported Clinical Pain and Psychological Characteristics Associated with Case Status

Compared to HC, women with VBD+IBS displayed greater levels of affective, aching, tender, and stabbing pain (Table 4.2). Women with VBD+IBS also demonstrated decreases in perceived general and physical health status, while women with VBD demonstrated lower

perceived mental health status. Additionally, VBD+IBS subjects reported more somatization. Lastly, women with VBD+IBS demonstrated a greater number of headaches and impact of pain on daily activity in the past 6 months. In sum, VBD subjects were similar to HC in self-reported pain and psychological characteristics, whereas VBD+IBS subjects reported greater clinical pain and decreased perceived health. Although not tabulated, no differences in SCL-90R subscale scores were observed between groups. Significance is $p<0.05$ and overall p-values for all variables are reported (Table 4.2).

4.3.5 Cytokines Associated with Case Status

Circulating cytokine levels were measured in subjects to determine if inflammatory mediators correlate with case status. Of the cytokines measured, IL-8 and IL-1ra were statistically significant between groups. Of the remaining 20 cytokines, 9 failed to exhibit differences between groups (MCP-1, MIP-1β, ENA-78, FGF basic, G-CSF, IL-6, TNFα, Tpo, and VEGF) and 11 were undetectable (MIP-1α, RANTES, GM-CSF, IFN-γ, IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-10, and IL-17). Compared to HC, women with VBD and VBD+IBS had increased expression of pro-inflammatory cytokine IL-8 ($F_{2,79}=6.337$, $p=0.0028$; Figure 4.4A). Women with VBD, but not with VBD+IBS, displayed a compensatory increase in anti-inflammatory cytokine IL-1ra (Figure 4.4B). These data suggest that women with VBD+IBS, but not VBD alone, have an impaired anti-inflammatory response.

4.3.6 miRNAs Associated with Case Status

We measured miRNA expression profiles to determine if miRNAs map onto distinguishing clinical features of IPDs. Compared to HC, VBD subjects had downregulation of 7 (miR-449b, miR-34b, miR-645, miR-503, miR-485-5p, miR-1294, miR-520f) and upregulation of 3 miRNAs (miR-200b, miR-133b, miR-520D-3p) at p-value<0.05 (Figure 4.5A). VBD+IBS subjects had downregulation of 6 (miR-1825, miR-1288, miR-593, miR-485-5p, miR-1294, miR-520f) and upregulation of 5 miRNAs (let-7f-2#, miR-512-3p, miR-

125a-3p, miR-661, miR-520D-3p) (Figure 4.5B). Downregulated miRNAs (fold change < -2) are depicted by red dots and upregulated miRNAs (fold change > 2) by blue dots (Figure 4.5A-B). All miRNAs are listed with corresponding fold change and p-value. miRNAs listed under “Both” were dysregulated in VBD and VBD+IBS subjects (Figure 4.5C). Results were verified for 5 of the dysregulated miRNAs using RT-PCR (Figure 4.6).

Specific miRNAs were associated with pain-relevant cytokines and intermediate phenotypes, with the direction of correlation varying according to miRNA and phenotype (Table 4.3) Specifically, 3 miRNAs were associated with IL-1ra expression and 2 miRNAs were associated with IL-8 expression. MiRNAs were also associated with self-reported pain and function, such that 12 miRNAs were associated with ‘stabbing pain’, 1 miRNA was associated with ‘affective pain’ and 2 miRNAs were associated with impact of pain on daily activity. Finally, miRNAs were associated with experimental pressure pain, such that 9 miRNAs were associated with pressure applied to the TMJ and 11 miRNAs were associated with pressure applied to the trapezius. Some miRNAs (miR-645, RNU44, miR-543, and miR-213) were associated with more than one phenotype and, thus, may play a key role in pain-relevant processes. All correlations with absolute values >0.40 and $p<0.05$ are reported (Table 4.3).

4.3.7 miRNA Pathways Associated with Case Status

We performed *in silico* pathway analysis using DIANA-miRPath v2.0 to identify genes and downstream pathways affected by miRNA dysregulation in IPDs.[320] In both groups, miRNA dysregulation is predicted to disrupt transforming growth factor beta (TGF-β), mitogen-activated protein kinase (MAPK) and Wnt signaling pathways (Figure 4.7, green). For example, upregulation of miR-520D-3p causes decreased levels of SMADs in the TGF- β pathway. Downregulation of miR-485-5p and miR-520f causes increased levels of MAPK genes such as protein kinase B (AKT1) and mitogen-activated protein 3-kinase 14

(MAP3K14). Downregulation of miR-485-5p results in increased levels of myc-associated factor X (MAX), another MAPK gene. Upregulation of miR-520D-3p causes increased levels of deubiquitinating enzymes (DUSPs), which act as MAPK phosphatases.[321] MAPKs and SMADs are also involved in Wnt signaling.

In VBD, but not VBD+IBS, the dorsal-ventral axis formation, gonadotropin-releasing hormone (GnRH), and gap junction pathways may be affected (Figure 4.7, blue). For example, downregulation of miR-34b and miR-449b leads to increased levels of dorsal-ventral axis proteins including receptor tyrosine-protein kinase erbB-4 (ERBB4), protein C-ets-1 (ETS1), and RAF proto-oncogene serine/threonine-protein kinase (RAF1). Downregulation of miR-200b causes increased levels of many gap junction proteins including: epidermal growth factor receptor (EGFR), GTPase KRas (KRAS), adenylate cyclase type 9 (ADCY9), phospholipase C beta-4 (PLCB4), cAMP-dependent protein kinase catalytic subunit beta (PRKACB), protein kinase C (PRKCA), and lysophosphatidic acid receptor 1 (LPAR1). Many of these proteins (*i.e.,* RAF1, ADCY9, KRAS, PLCB4, PRKACB, and PRKCA) are also vital to the GnRH pathway.

In VBD+IBS, but not VBD, the extracellular matrix (ECM), insulin resistance, and dentatorubral-pallidoluysian atrophy (DRPLA) pathways may be affected (Figure 4.7, red). Downregulation of miR-593 causes increased expression of ECM proteins including fibronectin type III domain containing 1 (FNDC1) and integrin alpha 5 (ITGA5). Upregulation of let-7f and miR-661 causes decreased levels of ECM proteins alpha-1 type I collagen (COL1A1) and dystroglycan 1 (DAG1), respectively. Upregulation of miR-125a-3p and miR-512-3p causes decreased levels of integrin beta 1 (ITGB1), another ECM protein; and insulin (INS), insulin receptor (INSR), and phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (PIK3CA), each of which is involved with insulin resistance. The insulin resistance and DRPLA pathways greatly overlap, sharing proteins such as INS and INSR. Full lists of the top 20 affected pathways for each group are reported with names and

union –ln(p-values) for target genes (Tables 4.4 and 4.5). Shared and unique pathways identified in VBD versus VBD+IBS may provide insight on the mechanisms underlying localized and widespread pain.

4.4 Discussion

IPDs are heterogeneous in nature and have limited effective therapeutic options. Here, we identify two subtypes of chronic pain patients, those with localized pain (VBD) and those with widespread pain (VBD+IBS) that differ with respect to pain phenotypes, psychological profiles, circulating cytokine expression and miRNA profiles. Pathway analysis of miRNA targets further suggests mechanisms that are common and unique to VBD and VBD+IBS.

Historically, VBD has been defined as a vulvar mucosa condition.[306] Our results, however, demonstrate that enhanced pelvic pressure pain in VBD and VBD+IBS is experienced predominantly in the muscles, suggesting modest increases in mucosal pain may exist as a byproduct of muscle pain. These data are in line with recent evidence that proposes a complex pathophysiology for VBD, involving the muscles as well as the peripheral and central nervous systems.[322] This suggests that VBD is, and should be treated as, a musculoskeletal pain condition.

Although VBD and VBD+IBS groups had similar pelvic pain phenotypes, they differed in remote bodily pain phenotypes and psychological profiles. Increased responses to mechanical and thermal stimuli at remote bodily sites in VBD+IBS indicates central enhancement in the processing of pain-evoking sensations.[40] Our results are in line with those from studies that suggest chronic pain conditions such as VBD likely reflect localized sensory dysregulation in the affected area[323] whereas widespread bodily pain often involves dysregulation in central pain processing, perhaps due to sensitization of spinal

nociceptors[324] or impairments in descending noxious control systems.[325] This probably explains why there was co-occurrence of TMD or FM for several of the VBD+IBS subjects and it raises the possibility that those, or other, overlapping pain conditions might likewise be associated with circulating cytokine expression and miRNA profiles. Unfortunately, there were too few TMD and FM subjects to address that question with sufficient power in this study, although it merits investigation in future studies.

Women with VBD also differed from those with VBD+IBS in circulating cytokine expression. Women with VBD had elevated levels of pro-inflammatory cytokine IL-8 and anti-inflammatory cytokine IL-1ra. This is consistent with studies that have demonstrated elevated inflammatory cytokine levels in pain conditions such as TMD and FM.[326,327] Women with VBD+IBS had elevated levels of IL-8 with no compensatory increase in IL-1ra. IL-1ra, a negative regulator of inflammation, can suppress IL-8 expression and block IL-8-induced mechanical pain.[328,329] Our finding is similar to that of a study that indicates an imbalance between IL-8 and IL-1ra in individuals with TMD plus widespread pain.[32] In sum, women in this study can be divided into two subsets: (1) those with localized pelvic pain, whose pain at other bodily sites resembles that of HC, who otherwise report good health, and who have intact anti-inflammatory responses; and (2) those with co-occurring pain conditions, more pain at remote bodily sites, poorer self-reported health, and impaired anti-inflammatory responses.

As miRNAs are key regulators of processes related to pain, psychological variables, and inflammatory responses, we explored their expression in VBD and VBD+IBS by miRNA profiling. Shared dysregulation of miRNAs may help to elucidate the mechanisms underlying IPDs. Both VBD and VBD+IBS groups in this study demonstrate dysregulation of miR-485-5p, miR-1294, miR-520f, and miR-520D-3p. Previous studies have found the same miRNAs to be associated with other diseases. For example, miR-485-5p[330,331] and miR-520D-3p[332] have been linked with cancer, and miR-1294 has been linked to Alzheimer's [333] and

Parkinson's disease.[334] While little is understood about the function and role of miR-520f in human disease, the miR-520 family is known to regulate IL-8.[335] Downregulation of miR-520f, but not upregulation of miR-520D-3p, can explain the increased IL-8 levels measured here in women with VBD and VBD+IBS. It is important to note that miRNA regulation is dynamic and complex. MiRNA expression can be affected by the presence of downstream mRNAs or proteins, and can be indirectly influenced by miRNAs within the same family. Several interactions, particularly in the form of negative or positive feedback loops, have been established within miRNA families.[336] Additional experiments are necessary to confirm the relationship between the miR-520 family and IL-8 in VBD and VBD+IBS, and to measure the concerted effect of these and other miRNAs on cytokine expression.

To further understand how these miRNAs could contribute to IPDs, we performed pathway analysis of predicted downstream targets. Both the VBD and VBD+IBS groups share predicted disruption of pain-relevant pathways. For example SMADs, which are downregulated in IPDs, are responsible for regulating anti-inflammatory cytokines of the TGF-β family and controlling inflammatory response.[337,338] Our results are in line with those from the TMD study previously mentioned that demonstrates suppression of the TGF-β pathway in individuals with either TMD or TMD plus widespread pain.[32] We demonstrate potential upregulation of AKT1, MAP3K14, MAX, and DUSPs in IPDs, each of which may contribute to pain sensitization, pro-inflammatory mediator synthesis and the development of chronic pain *via* the MAPK pathway.[109,321,339-341] MAPKs and SMADs are also involved in Wnt signaling, a pathway that alters production of pro-inflammatory cytokines and sensitivity of peripheral sensory neurons, thereby contributing to development of neuropathic pain.[342,343] We conclude that dysregulation of miRNAs and downstream pathways may promote a constant state of inflammation and pain in women with VBD and VBD+IBS.

Dysregulated miRNAs that do not overlap between the two groups may represent unique pathways of vulnerability. Women with VBD have dysregulation of miR-449b, miR-

34b, miR- 645, miR-503, miR-200b, and miR-133b. MiR-449b,[344] miR-34b,[345] miR-645,[346] miR-503[347],miR-200b,[348] and miR-133b [349] have been implicated in cancer. Notably, miR-449b is known to regulate neurokinin-1 receptor (NK1R) in chronic bladder pain syndrome (BPS). [350] The NK1 pathway is important for pain transmission and neuroimmune modulation[351] and may be affected similarly in VBD and BPS.

Based upon miRNA dysregulation, our pathway analysis predicts that women with VBD have increased levels of ERBB4, ETS1, and RAF1, each of which may contribute to inflammation or the development of neuropathic pain.[352-355] Other proteins targeted in VBD include EGFR, KRAS, ADCY9, PLCB4, PRKACB, PRKCA, and LPAR1, which are all linked to inflammation.[356-358] Interestingly, another common denominator of the genes and pathways affected in VBD is estrogen.[359-363] RAF1, ADCY9, KRAS, PLCB4, PRKACB, and PRKCA are vital to the GnRH pathway, in which neuropeptide GnRH interacts with estrogen *via* a negative feedback loop to maintain hormone levels.[364] Other affected targets, such as ETS1, ERBB4, and LPAR1 interact directly with estrogen or estrogen receptors.[360,365,366] Our results predict that women with VBD will have blunted estrogen and GnRH activity. This provides a possible explanation for why estrogen and GnRH effectively reduce pain in endometriosis and other pelvic pain conditions.[367,368] From these results we conclude that miRNA dysregulation of estrogen-relevant genes contributes to localized pelvic pain characteristic of VBD.

We found that subjects with VBD+IBS show dysregulation of miR-1825, miR-1288, miR-593, let-7f-2#, miR-512-3p, miR-125a-3p, and miR-661. MiR-1825,[369] miR-593,[370] let-7f-2#,[371] miR-512-3p,[372] miR-125a-3p,[373] and miR-661[374] have all been shown to contribute to cancer. In addition, miR-1825 has been associated with avian influenza;[375] miR-1288 with ectopic pregnancy;[376] and let-7f-2# with lupus.[377] Most relevant to our results, miR-125a-3p has been shown to promote orofacial pain through upregulation of p38 MAPK.[378]

In VBD+IBS, our pathway analysis predicts that miRNA dysregulation will affect

genes of the ECM including COL1A1, ITGB1, and ITGA5. Alterations in the ECM, which provides structural and mechanical support to surrounding tissue, have been previously linked to many painful conditions.[379,380] INS and INSR, also affected in VBD+IBS, have been implicated in nociception *via* the insulin resistance pathway.[381] In addition, each of the pathways and molecules affected by miRNA dysregulation in VBD+IBS share an involvement in both muscle function and sensory processing. For example, COL1A1 has been implicated in skeletal muscle atrophy.[382] Decreases in ITGB1, PIK3CA, and INS have all been linked to impairments in muscle development and function.[383-385] ITGA5 interacts with fibronectins to influence astrocyte physiology[386] and ITGB1 is necessary for normal axon formation.[387] Decreased DAG1 expression is associated with abnormal myelin sheath folding and muscle impairment.[388] In sum, dysfunction of pathways such as muscle and sensory nerve processes may lead to central sensitization and generalized body pain in VBD+IBS.

While the present study did not enroll women with IBS alone, an emerging literature demonstrates the contribution of miRNAs, particularly the miR-29 family, to IBS.[389,390] Interestingly, we did not observe an overlap between the miRNAs associated with IBS in other cohorts and those associated with VBD or VBD+IBS in our cohort. This might reflect the differential expression of miRNAs in different sample types (*e.g.,* whole blood used in the present study versus colon tissue and microvesicles commonly used in IBS studies). Further, it might indicate that the pathophysiological processes that drive IBS are distinct from those that drive VBD or VBD+IBS.

In conclusion, we presented potentially separate pathways for localized versus widespread pain as predicted by miRNA profiles. miRNA dysregulation in VBD is predicted to affect estrogen-relevant pathways, explaining why pain is localized to the pelvis. In contrast, miRNA dysregulation in VBD+IBS may be linked to alterations in muscle, nerve, and glial cell function, thereby contributing to widespread pain. While these results suggest

that miRNAs hold promise as biomarkers for chronic pain, it is important to note that existing miRNA studies of human IPDs are underpowered statistically and our knowledge of miR-1294, miR-645, miR-1825, and miR-1288 is extremely limited. Further studies are required to 1) identify miRNA profiles in a larger population of chronic pain patients; 2) observe miRNA expression profiles in sample types other than whole blood (*e.g.,* plasma, tissue, specific cell types); 3) validate changes in the expression levels of the predicted targets (*e.g.,* estrogen and insulin); 4) correlate miRNA profiles with response to treatments that target different pathophysiologic processes (*e.g.,* topical estrogen therapy to improve local vestibular tone[391] versus anticonvulsant medication to calm the activity of nociceptive neurons[392-394]); and 5) assess the effectiveness of targeting miRNAs and downstream pathways to reduce and manage symptoms of chronic pain patients. Accomplishing these next steps will facilitate the use of miRNA screening tools in the clinic to determine the most effective treatment with minimal risk for patients with chronic pain. Such studies should help inform the development of novel therapeutics targeted against one of several key elements along the canonical pathway from gene to protein.

4.5 Acknowledgements

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4.6 Footnotes

[1]Reprinted from *Translational Research,* S1931-5244(15)00213-3, Ciszek B, Khan A, Dang H, Slade G, Smith S, Bair E, Maixner W, Zolnoun D, Nackley A, MicroRNA expression profiles differentiate chronic pain condition subtypes, Copyright (2015); with permission from Elsevier.[395] Final Text Available Online at:

http://www.ncbi.nlm.nih.gov/pubmed/?term=ciszek+microrna

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Table 4.1 Demographic Data

Demographic Data	HC	VBD	VBD+IBS
N	22	33	23
Age	26.09 (1.04)	28.06 (1.10)	28.13 (1.36)
Weight (kg)	69.90 (0.66)	63.63**** (0.45)	60.88**** (0.66)
RACE			
White	16	25	16
Black	2	4	2
Hispanic	1	1	0
Other	3	3	5
EDUCATION			
High school	1	1	1
Some college	3	4	4
College grad	6	13	10
Post grad	12	15	8
INCOME			
0-39k	7	12	10
40-79k	7	9	6
80-149k	2	6	2
150k+	3	2	1
refused or left blank	3	4	3
COMORBID CONDITIONS			
VBD + IBS only	N/A	N/A	15
VBD + IBS + TMD	N/A	N/A	4
VBD + IBS + TMD + FM	N/A	N/A	4

Abbreviations: healthy control (HC), vestibulodynia (VBD), irritable bowel syndrome (IBS), temporomandibular joint disorder (TMD), fibromyalgia (FM). For age and weight, data are mean (SEM). For other categories, N is reported for each group. ****p<0.0001 compared to HC.

Table 4.2 Self-reported pain, function, and psychological characteristics associated with case status

Phenotype	Questionnaire	HC	VBD	VBD + IBS	Adjusted P-value
Affective Pain	MPQ	11.67 (0.41)	12.50 (0.62)	14.78*** (0.8)	0.001
Aching Pain	MPQ	1.14 (0.10)	1.23 (0.10)	2.06†††† (0.17)	<0.0001
Tender Pain	MPQ	1.10 (0.06)	1.37 (0.13)	1.61** (0.16)	0.003
Stabbing Pain	MPQ	1.00 (0.00)	1.13 (0.10)	1.39* (0.20)	0.038
General Health	SF12v2	4.51 (0.11)	4.31 (0.15)	4.01* (0.15)	0.012
Mental Health	SF12v2	51.82 (1.87)	45.01* (1.96)	46.41 (2.20)	0.069
Physical Health	SF12v2	55.83 (1.12)	57.58 (0.85)	50.54†† (1.26)	0.003
Somatization	PILL	89.79 (4.15)	98.44 (3.60)	115.61†† (4.61)	0.0002
Headache Types	CPSQ	1.37 (0.19)	1.93 (0.19)	2.37** (0.23)	0.009
Impact of Pain on Daily Activity	CPSQ	0.30 (0.20)	0.81 (0.37)	2.05* (0.64)	0.028

Data are expressed as Mean (SEM). *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 compared to HC. ††p<0.01, ††††p<0.0001 as compared to HC and VBD.

Table 4.3 MicroRNA expression is correlated with intermediate phenotypes

Intermediate Phenotype	miRNA	Correlation (r)	Adjusted p-value
IL-1ra expression	miR-99b	-0.74	<0.0001
	miR-373	0.44	0.020
	miR-627	0.42	0.034
IL-8 expression	miR-125a-5p	-0.44	0.052
Stabbing Pain (MPQ)	miR-1305	0.56	<0.001
	miR-425#	-0.54	<0.001
	miR-30d	-0.54	<0.001
	miR-1255B	-0.52	<0.001
	miR-454#	-0.51	<0.001
	miR-302b	0.51	<0.001
	miR-15b#	-0.51	<0.001
	miR-320B	-0.49	<0.01
	miR-551b	0.43	0.015
	miR-570	0.42	0.023
	miR-1254	-0.41	0.027
	miR-487a	0.40	0.037
Affective Pain (MPQ)	miR-551b	0.49	0.015
Impact of Pain on Daily Activity (CPSQ)	miR-491-3p	0.48	0.029
	miR-10b	0.46	0.035
Remote Bodily Pressure Pain (Masseter)	RNU44	-0.55	<0.001
	miR-645	-0.47	0.010
	miR-1274A	-0.45	0.016
	miR-213	-0.42	0.036
	miR-543	-0.41	0.037
	RNU48	-0.41	0.037
	miR-192#	-0.40	0.041
	miR-1274B	-0.40	0.041
Remote Bodily Pressure Pain (Trapezius)	RNU44	-0.56	<0.001
	RNU48	-0.44	0.019
	miR-543	-0.44	0.019
	miR-645	-0.44	0.019
	miR-1274A	-0.43	0.019
	miR-1270	-0.43	0.019
	miR-1179	-0.43	0.019
	miR-589	-0.42	0.020
	miR-378	-0.42	0.022
	miR-213	-0.41	0.024
	miR-1180	-0.40	0.027

Abbreviations: Interleukin 1 receptor antagonist (IL-1ra), interleukin 8 (IL-8), McGill Pain Questionnaire (MPQ), Comprehensive Pain and Symptom Questionnaire (CPSQ).

Table 4.4 MicroRNA Pathway Dysregulation in VBD

MiRNAs	Target Genes	ln(p-val)
	Wnt Signaling	
miR-34b **miR-449b** **miR-503** **miR-645** **miR-200b** **miR-133b**	CCND2, CTNNB1, DKK1, FZD4, FZD5, MAP3K7, VANGL2, DAAM1, LEF1, MYC, NFAT5, PLCB1, SMAD2, SMAD4, TP53, WNT1, BTRC, CCND2, CCND2, CCND3, FOSL1, MAP3K7, NKD1, PPP3CB, WNT3A, PPP3CB, CTBP2, EP300, FBXW11, JUN, MPAK9, PLCB4, PPP2R2C, PRKACB, PRKCA, RAC1, RHOA, SIAH1, WNT16, CTBP2, CXXC4, FBXW11, NFAT5, NFATC2, PLCB4, PPP2CA, PPP2CB, PSEN1, TBL1X, TCF7, WNT4	21.37
	Adherens Junction	
miR-34b **miR-449b** **miR-503** **miR-200b** **miR-133b**	CTNNB1, MAP3K7, LEF1, MET, PTPRM, PVRL1, SMAD2, SMAD4, VCL, WASF1, IGF1R, MAP3K7, PVRL1, WASL, EP300, LMO7, PVRL1, PVRL4, RAC1, RHOA, WASF1, WASF3, EGFR, FGFR1, IGF1R, INSR, MLLT4, TCF7, TGFBR1, WASF2, YES1	18.76
	Colorectal Cancer	
miR-34b **miR-449b** **miR-503** **miR-200b** **miR-133b**	CTNNB1, FZD4, FZD5, GRB2, PIK3R3, LEF1, MAP2K1, MET, MYC, PDGFRA, SMAD2, SMAD4, TP53, AKT3, BCL2, CCND1, CDD, IGF1R, MAP2K1, PIK3R1, RAF1, ACVR1C, APPL1, BCL2, JUN, KRAS, MAPK9, RAC1, SOS1, EGFR, IGF1R, TCF7, TGFBR1	18.33
	Prostate Cancer	
miR-34b **miR-449b** **miR-503** **miR-200b** **miR-133b**	CREB1, CREB3, CREB3L1, CTNNB1, GRB2, PDGFA, PIK3R3, CREB1, CREB3L1, E2F3, LEF1, MAP2K1, PDGFRA, TP53, AKT3, BCL2, CCND1, CCNE1, CREB5, E2F3, IGF1R, IKBKB, MAP2K1, PIK3RI, RAF1, BCL2, CCNE2, CDK2, CDKN1B, CREB5, E2F3, EP300, IKBKB, KRAS, SOS1, CREB5, EGFR, FGFR1, IGF1R, TCF7	17.01
	Chronic Myleoid Leukemia	
miR-34b **miR-449b** **miR-503** **miR-200b** **miR-133b**	GRB2, PIK3R3, E2F3, HDAC1, MAP2K1, MYC, SMAD4, TP53, AKT3, CCND1, E2F3, IKBKB, MAP2K1, PIK3R1, RAF1, ACVR1C, CBL, CDKNN1B, CRKL, CTBP2, E2F3, IKBKB, KRAS, PTPN11, SHC1, SOS1, BCL2L1, CRK, CTBP2, EVI1, TGFBR1	14.95
	Renal Cell Carcinoma	
miR-34b	GRB2, PIK3R3, RAP1B, ARNT2, ETS1, MAP2K1, MET, AKT3,	14.35

miR-449b miR-503 miR-200b miR-133b	MAP2K1, PIK3R1, RAF1, VEGFA, CRKL, EGLN1, EP300, ETS1, GAB1, JUN, KRAS, PAK6, PAK7, PTPN11, RAC1, RAP1B, SOS1, TCEB1, VEGFA, CRK	
ErbB Signaling Pathway		
miR-34b miR-449b miR-503 miR-645 miR-200b miR-133b	ERBB4, GRB2, NCK2, PIK3R3, RPS6KB1, MAP2K1, MYC, RPS6KB1, AKT3, MAP2K1, PIK3R1, RAF1, HBEGF, CBL, CDKN1B, CRKL, GAB1, JUN, KRAS, MAPK9, PAK6, PAK7, PLCG1, PRKCA, RPS6KB1, SHC1, SOS1, CRK, EGFR, MAP2K4	12.44
Melanogenesis		
miR-34b miR-449b miR-503 miR-200b miR-133b	CREB1, CREB3, CREB3L1, CTNNB1, FZD4, FZD5, CREB1, CREB3L1, KITLG, MAP2K1, MITF, PLCB1, WNT1, GNAI3, MAP2K1, RAF1, WNT3A, ADCY2, ADCY9, EP300, GNAI3, KRAS, PLCB4, PRKACB, PRKCA, WNT16, ADCY5, ADCY6, CALM1, PLCB4, TCF7, WNT4	10.51
Focal Adhesion		
miR-34b miR-449b miR-503 miR-200b miR-133b	CCND2, CTNNB1, GRB2, ITGA2, PDGFA, PIK3R3, RAP1B, RELN, VAV3, MAP2K1, MET, PDGFRA, THBS1, VCL, AKT3, BCL2, CCND1, CCND2, CCND3, IGF1R, MAP2K1, MYLK, PIK3R1, RAF1, VEGFA, BCL2, CRKL, FLT1, FM1, JUN, KDR, LAMC1, MAPK9, MYLK, PAK6, PAK7, PRKCA, RAC1, RAP1B, RELN, RHOA, SHC1, SOS1, TLN2, VEGFA, XIAP, COL1A1, COL5A3, COL6A3, CRK, EGFR, IGF1R, TNR	10.21
MAPK Signaling Pathway		
miR-34b miR-449b miR-503 miR-645 miR-200b miR-133b	CACNB2, GRB2, MAP3K1, MAP3K7, MAP3K7IP2, PDGFA, RAP1B, CACNB1, CACNB3, FGF23, HSPA1B, MAP2K1, MAP4K4, MYC, PDGFRA, RPS6KA4, RRAS, TAOK1, TP53, AKT3, FGF2, FGF7, IKBKB, MAP2K1, MAP3K7, MAPK8IP2, NF1, PPP3CB, PTPRR, RAF1, MEF2C, PPP3CB, ACVR1C, CACNA2D1, CACNB2, CRKL, DUSP1, IKBKB, JUN, KRAS, MAP2K5, MAP3K1, MAP3K5, MAP4K3, MAP4K4, MAPK9, NTF3, PRKACB, PRKCA, RAC1, RAP1B, RPS6KA3, SOS1, SRF, CRK, DUSP1, EGFR, EV1, FGF1, FGFR1, MAP2K4, MAP3K3, NFATC2, TGFBR1	10
Regulation of Actin Cytoskeleton		
miR-34b miR-449b miR-503 miR-	CFL2, ITGA2, NCKAP1, PDGFA, PIK3R3, PIP5K1B, PIP5K3, VAV3, FGF23, IQGAP2, MAP2K1, MYH9, PDGFRA, RDX, RRAS, VCL, WASF1, FGF2, FGF7, MAP2K1, MYH10, MYLK, PIK3R1, RAF1, WASL, CFL2, CRKL, FGD1, FN1, KRAS, LIMK1, MSN, MYLK, PAK6, PAK7, PIP4K2B, PIP5K3, PPP1R12B, RAC1,	9.67

200b miR- 133b	RHOA, SOS1, SSH2, WASF1, ARPC1A, ARPC5, CRK, DIAPH2, EGFR, FGF1, FGFR1, IQGAP2, MSN, MYH9, PFN2, PIP4K2B, PIP5K3, WASF2	
Pancreatic Cancer		
miR-34b miR- 449b miR-503 miR- 200b miR- 133b	PIK3R3, E2F3, MAP2K1, RALA, SMAD2, SMAD4, TP53, AKT3, CCND1, E2F3, IKBKB, MAP2K1, PIK3R1, RAF1, VEGFA, ACVR1C, BRCA2, E2F3, IKBKB, KRAS, MAPK9, RAC1, VEGFA, BCL2L1, EGFR, JAK1, TGFBR1	9.57
Axon Guidance		
miR-34b miR- 449b miR-503 miR-645 miR- 200b miR- 133b	CFL2, DPYSL2, EPHA7, NCK2, NTNG1, ABLIM3, EFNB1, MET, NFAT5, SEMA4C, SEMA4F, DCC, EFNB2, EPHA7, GNAI3, PPP3CB, SEMA3D, SEMA6D, PPP3CB, SEMA3F, SEMA6D, CFL2, EFNA1, EFNB2, GNA13, KRAS, LIMK1, PAK6, PAK7, PLXNA2, RAC1, RHOA, SEMA3F, SEMA6D, EFNA4, EPHA7, EPHB4, NFAT5, HFATC2, SRGAP2, SRGAP3	8.58
Notch Signaling Pathway		
miR- 449b miR-503 miR- 200b miR- 133b	APH1A, DLL1, HDAC1, JAG1, NCSTN, NOTCH2, NUMBL, NUMB, CTBP2, EP300, JAG2, KAT2B, NUMB, CTBP2, PSEN1, RBPJ	8.32
Thyroid Cancer		
miR-34b miR- 449b miR-503 miR- 200b miR- 133b	CCDC6, CTNNB1, LEF1, MAP2K1, MYC, RET, TP53, CCND1, MAP2K1, KRAS, TCF7, TFG	8.21
Glioma		
miR-34b miR- 449b miR-503 miR- 200b miR- 133b	GRB2, PDGFA, PIK3R3, E2F3, MAP2K1, PDGFRA, TP53, AKT3, CCND1, E2F3, IGF1R, MAP2K1, PIK3R1, RAF1, E2F3, KRAS, PLCG1, PRKCA, SHC1, SOS1, CALM1, EGFR, IGF1R	8.06
TGF-Beta Signaling Pathway		
miR-34b miR-	ACVR2A, ID2, RP26KB1, SMURF1, ACVR2B, E2F5, MYC, RPS6KB1, SMAD2, SMAD4, THBS1, ACVR2A, ACVR2B,	7.82

449b **miR-503** **miR-200b** **miR-133b**	BMPR1A, SMAD7, SMURF1, SMURF2, ACVR1C, ACVR2A, EP300, NOG, PPP2R2C, RHOA, RPS6KB1, SMURF2, ID4, LTBP1, PPP2CA, PPP2CB, SP1, TGFBR1	
Acute Myeloid Leukemia		
miR-34b **miR-449b** **miR-503** **miR-200b** **miR-133b**	GRB2, PIK3R3, RPS6KB1, LEF1, MAP2K1, MYC, RPS6KB1, AKT1, CCND1, IKBKB, MAP2K1, PIK3R1, RAF1, IKBKB, KRAS, PIM2, RPS6KB1, SOS1, JUP, PML, TCF7	7.67
Oxidative Phosphorylation		
miR-449b **miR-200b** **miR-133b**	SDHC, NDUFS4, NDUFS3	7.21
Dorso-Ventral Axis Formation		
miR-34b **miR-449b** **miR-503** **miR-200b** **miR-133b**	ERBB4, GRB2, ETS1, MAP2K1, NOTCH2, MAP2K1, RAF1, ETS1, ETS2, KRAS, SOS1, EGFR	7.18

The top 20 pathways, as determined by the Diana Lab DNA Intelligent Analysis, affected by miRNA dysregulation in women with VBD are shown with the names and union –ln(p-value) of target genes affected in each pathway. Genes are linked to miRNAs by color.

Table 4.5 MicroRNA Pathway Dysregulation in VBD+IBS

MiRNAs	Target Genes	ln(p-val)
	MAPK Signaling Pathway	
miR-593 let-7f-2# miR-125a-3p miR-512-3p miR-661	PDGFA, PDGFB, PTPN5, PTPRR, RAP1B, RPS6KA1, TAOK3, TGFB2, ACVR1B, ACVR1C, CACNA1D, CASP3, DUSP16, DUSP1, DUSP4, DUSP9, FGF11, FGF5, FLNA, MAP3K1, MAP3K3, MAP3K7IP2, MAP4K3, MAP4K4, MAPK11, MAPK8, NGF, NLK, PAK1, PDGFB, PPP3CA, RPS6KA3, TGFBR1, TP53, IL1R1, NF1, NLK, RPS6KA3, AKT1, CRK, DUSP1, EVI1, FGF19, MAP3K14, MAP3K1, MAPK10, MEF2C, MKNK2, PAK2, PPP3CA, RPS6KA2, RPS6KA3, RPS6KA5, SOS1, TGFBR2, DUSP3, FLNA, MAP3K10, MAP3K3, PLA2G6	16.5
	TGF-Beta Signaling Pathway	
miR-593 let-7f miR-125a-3p miR-512-3p miR-661	ACVR1, BMPR2, NOG, SP1, TGFB2, ACVR1B, ACVR1C, ACVR2A, ACVR2B, CHRD, E2F5, GDF6, TGFBR1, THBS1, ZFYVE16, ACVR2B, BMPR2, CUL1, E2F5, INHBB, LEFTY1, LEFTY2, PITX2, RBL1, RBL2, SMAD2, TGFBR2, PPP2R1A	16.07
	Chronic Myeloid Leukemia	
miR-593 let-7f miR-125a-3p miR-512-3p miR-661	CDK6, CTBP2, SHC4, TGFB2, ACVR1B, ACVR1C, BCL2L1, CBL, CCND1, CDKN1A, RB1, TGFBR1, TP53, E2F3, ACVR1C, APPL1, BCL2, JUN, KRAS, MAPK9, RAC1, SOS1, EGFR, IGF1R, TCF7, TGFBR1	15.09
	Colorectal Cancer	
miR-593 let-7f miR-125a-3p miR-512-3p miR-661	CREB1, CREB3, CREB3L1, CTNNB1, GRB2, PDGFA, PIK3R3, CREB1, CREB3L1, E2F3, LEF1, MAP2K1, PDGFRA, TP53, AKT3, BCL2, CCND1, CCNE1, CREB5, E2F3, IGF1R, IKBKB, MAP2K1, PIK3RI, RAF1, AKT1, CCND1, CDK4, CRK, E2F3, EVI1, PIK3CA, RUNX1, SOS1, TGFBR2, CBL, CDK6	11.98
	Focal Adhesion	
miR-593 let-7f miR-125a-3p miR-512-3p miR-661	COL4A6, ITGA10, ITGA5, PDGFA, RAP1B, SHC4, CCND1, CCND2, COL11A1, COL1A1, COL1A2, COL3A1, COL4A1, COL4A6, COL5A2, FLNA, IGF1, IGF1R, ITGB3, MAPK8, PAK1, PDGFB, THBS1, VAV3, FYN, IGF1R, ITGB1, ACTG1, AKT1, CCND1, CCND2, CRK, FLT1, IGF1R, ITGB8, LAMA3, MAPK10, PAK2, PAK7, PIK3CA, SOS1, COL6A3, FLNA, ITGA10, KDR	11.35
	Axon Guidance	
miR-593 let-7f miR-125a-3p miR-512-3p miR-661	EPHB3, SRGAP3, EPHA4, EPHB1, LIMK2, PAK1, PPP3CA, SEMA4C, SEMA4F, SEMA4G, CFL2, DCC, FYN, ITGB1, SEMA5B, ABLIM1, CFL2, DPYSL5, EFNB2, EPHA2, EPHA8, NTN4, PAK2, PAK7, PLXNA1, PPP3CA, SEMA3C, GNAI2, SEMA4G, SLIT1, SRGAP3	10.33
	Pancreatic Cancer	
miR-593 let-7f	CDK6, TGFB2, ACVR1B, ACVR1C, BCL2L1, CCND1, MAPK8, RB1, TGFBR1, TP53, CASP9, E2F3, AKT1, CCND1, CDK4,	8.91

miR-125a-3p miR-512-3p miR-661	E2F3, MAPK10, PIK3CA, SMAD2, TGFBR2, CDK6	
	ECM Receptor Interaction	
miR-593 let-7f miR-125a-3p miR-512-3p miR-661	COL4A6, FNDC1, ITGA10, ITGA5, COL11A1, COL1A1, COL1A2, COL3A1, COL4A1, COL4A6, COL5A2, FNDC3A, ITGB3, THBS1, ITGB1, CD44, FNDC3A, LAMA3, COL6A3, DAG1, ITGA10	8.61
	Oxidative Phosphorylation	
miR-512-3p	ATP5E	6.81
	Glioma	
miR-593 let-7f miR-125a-3p miR-512-3p miR-661	CDK6, PDGFA, PDGFB, SHC4, CCND1, CDKN1A, IGF1, IGF1R, PDGFB, RB1, TP53, E2F3, IGF1R, AKT1, CCND1, CDK4, E2F3, IGF1R, PIK3CA, SOS1, CDK6	6.7
	Melanoma	
miR-593 let-7f miR-125a-3p miR-512-3p miR-661	CDK6, PDGFA, CCND1, CDKN1A, FGF11, FGF5, IGF1, IGF1R, PDGFB, RB1, TP53, E2F3, IGF1R, AKT1, CCND1, CDK4, E2F3, FGF19, IGH1R, PIK3CA, CDK6	6.27
	Adherens Junction	
miR-593 let-7f miR-125a-3p miR-512-3p miR-661	TCF7, ACVR1B, ACVR1C, IGF1R, INSR, NLK, TGFBR1, WASL, FYN, IGF1R, INSR, NLK, SNAI1, ACTG1, IGF1R, LEF1, SAMD2, SSX1P, TGFBR2, PVRL2, WASL	6.27
	Prostate Cancer	
miR-593 let-7f miR-125a-3p miR-512-3p	CREB3L1, PDGFA, TCF7, CCND1, CDKN1A, IGF1, IGF1R, INS, PDGFB, RB1, TP53, CASP9, E2F3, IGF1R, INS, AKT1, CCND1, CREB5, E2F3, IGF1R, LEF1, PIK3CA, SOS1	5.65
	Type II Diabetes	
let-7f miR-125a-3p miR-512-3p miR-661	CACNA1D, INSR, IRS2, MAPK8, SOCS1, SOCS4, INS, INSR, MAPK10, PIK3CA, SLC2A4, SOCS4	5.3
	mTOR Signaling Pathway	
miR-593 let-7f miR-125a-	RPS6KA1, IGF1, INS, RICTOR, RP26KA3, TSC1, ULK2, INS, RPS6KA3, AKT1, PIK3CA, RPS6KA2, RPS6KA3, ULK1, ULK2	4.77

3p miR-512-3p miR-661		
Dentatorubropallidoluysian Atrophy (DRPLA)		
miR-593 let-7f miR-125a-3p miR-512-3p	ITCH, CASP3, INSR, INS, INSR, MAGI1	4.75
Bladder Cancer		
let-7f miR-125a-3p miR-512-3p	CCND1, CDKN1A, IL8, RB1, THBS1, TP53, E2F3, CCND1, CDK4, DAPK2, E2F3, IL8, RPS6KA5	4.52
Wnt Signaling Pathway		
miR-593 let-7f miR-125a-3p miR-512-3p miR-661	CTBP2, FOSL1, FXD7, NKD1, PRICKLE2, TCF7, VANGL1, VANGL2, CCND1, CCND2, FZD4, MAPK8, NKD1, NLK, PPP3CA, SENP2, TP53, VANGL1, VANGL2, WNT1, CSNK1E, NLK, CCND1, CCND2, CUL1, LEF1, MAPK10, PPP3CA, SMAD2, FZD4, FZD8, PPP2R1A	4.08
ErbB Signaling Pathway		
miR-593 let-7f miR-125a-3p miR-512-3p miR-661	ERBB4, NRG3, SHC4, ABL2, CBL, CDKN1A, MAPK8, PAK1, NRG1, AKT1, CRK, MAPK10, PAK2, PAK7, PIK3CA, SOS1, CBL	3.75
Regulation of Actin Cytoskeleton		
miR-593 let-7f miR-125a-3p miR-512-3p miR-661	ITGA10, ITGA5, ITGB1, PDGFA, SCIN, DIAPH2, FGF11, FGF5, ITGB3, LIMK2, PAK1, PDGFB, RDX, SSH1, VAV3, WASL, CFL2, INS, ITGB1, ACTG1, CFL2, CRK, FGF19, ITGB8, PAK2, PAK7, PFN2, PIK3CA, RDX, SOS1, SSH2, ARHGEF4, ITGA10, PIP4K2B, WASL	3.65

The top 20 pathways, as determined by the Diana Lab DNA Intelligent Analysis, affected by miRNA dysregulation in women with VBD+IBS are shown with the names and union –ln(p-value) of target genes affected in each pathway. Genes are linked to miRNAs by color.

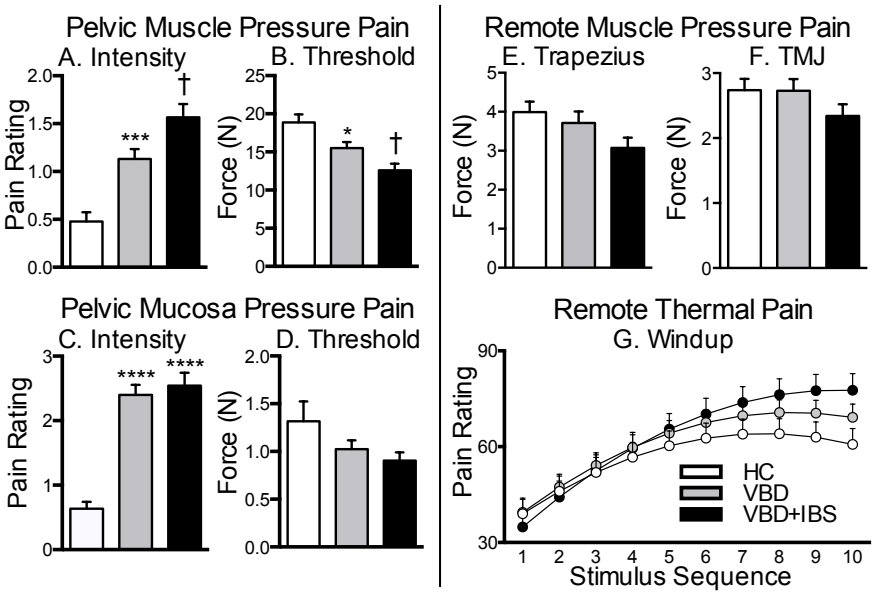


Figure 4.1 Pelvic muscle and mucosa pressure pain is enhanced in women with VBD and VBD+IBS, while remote bodily muscle pain and thermal windup is enhanced only in women with VBD+IBS. Women with VBD or VBD+IBS reported greater pain intensity and decreased pain thresholds in the pelvic muscle **(A-B)** and mucosa **(C-D)**. Women with VBD+IBS demonstrated a trend towards decreased pressure pain thresholds in the trapezius **(E)** and temporomandibular joint **(F)** as compared to HC and VBD. **(G)** For the thermal data, there was a significant interaction between study group and stimulus sequence, although not between study group and the square of stimulus sequence. VBD+IBS women exhibit the greatest degree of pain in response to the windup thermal heat assay. Data are Mean ± SEM. *p<0.05, ***p<0.001, ****p<0.0001 compared to HC; †p<0.05 as compared to HC and VBD. Abbreviations: healthy control (HC), irritable bowel syndrome (IBS), vestibulodynia (VBD), standard error of the mean (SEM), temporomandibular joint (TMJ).

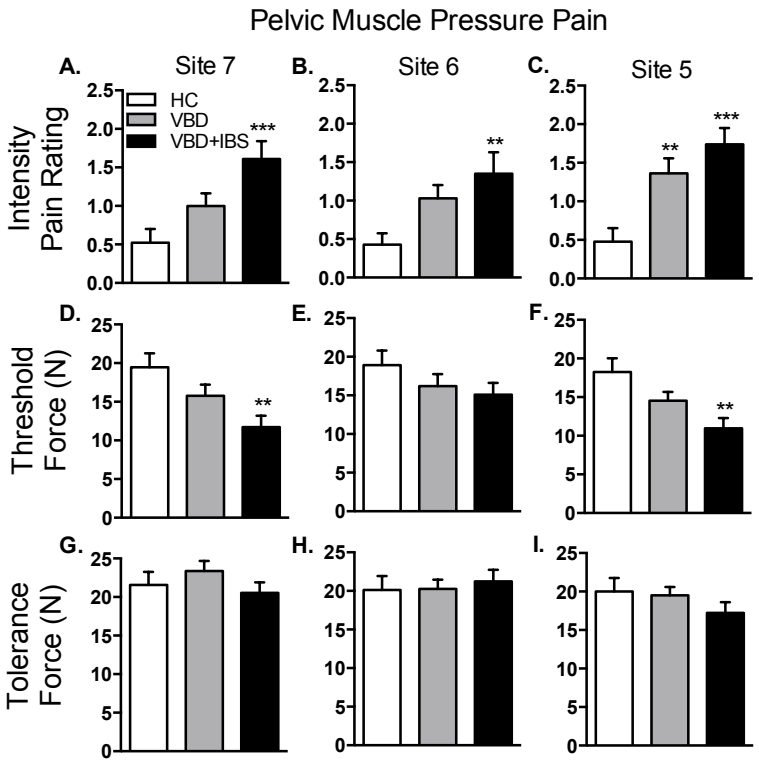


Figure 4.2 Pelvic muscle pressure pain is enhanced in patients with VBD and VBD+IBS. Patients with VBD alone exhibit modest increases in pain intensity **(A-C)** and decreases in mechanical threshold **(D-F)** at all 3 pelvic muscle sites, while those with VVS+IBS report dramatic increases in intensity and decreases in thresholds. No differences in tolerance were reported across groups **(G-I)**. Data are Mean ± SEM. *p<.05, **p<.01, ***p<.001 compared to HC. Abbreviations: healthy control (HC), irritable bowel syndrome (IBS), Newtons (N), vestibulodynia (VBD), standard error of the mean (SEM).

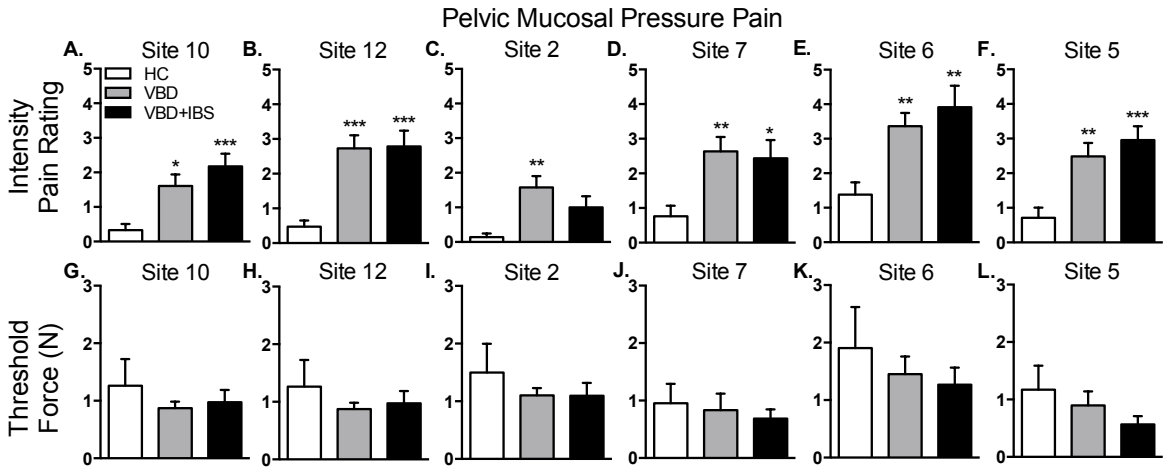


Figure 4.3 Pelvic mucosa pressure pain is enhanced in patients with VBD and VBD+IBS. VBD and VBD+IBS patients reported trends of enhanced pain intensity **(A-F)** and decreased pain thresholds **(G-L)** at all 6 pelvic mucosal sites. Data are Mean ± SEM. Abbreviations: healthy control (HC), irritable bowel syndrome (IBS), Newtons (N), vestibulodynia (VBD), standard error of the mean (SEM).

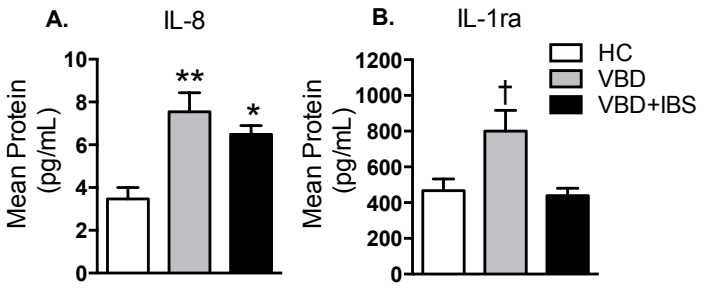
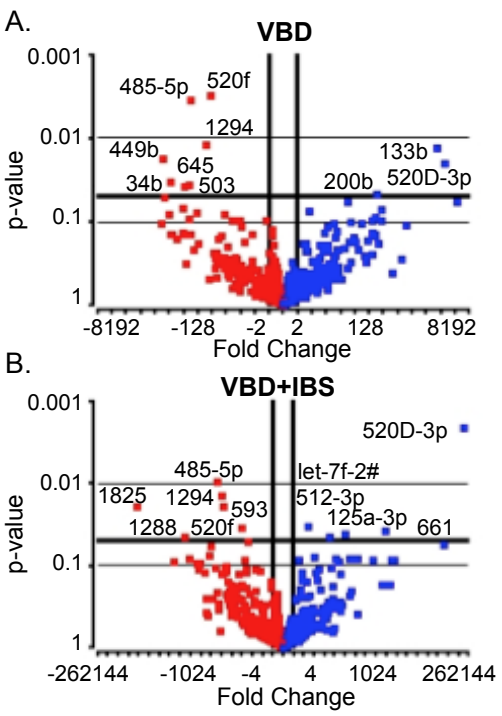


Figure 4.4 Cytokine expression is altered in women with VBD and VBD+IBS. Compared to HC, women with VBD exhibit elevated levels of pro-inflammatory cytokine IL-8 **(A)** and anti-inflammatory cytokine IL-1ra **(B)**. Women with VBD+IBS exhibit elevated levels of IL-8, but no compensatory increase in IL-1ra. Data are Mean ± SEM. *p<0.05, **p<0.01 compared to HC; †p<0.05 compared to HC and VBD+IBS. Abbreviations: healthy control (HC), interleukin (IL), irritable bowel syndrome (IBS), vestibulodynia (VBD), standard error of the mean (SEM).



C.

	miRNA	Fold Change	P-value
VBD	miR-449b	-330.14	0.017
	miR-34b	-239.45	0.003
	miR-645	-117.87	0.038
	miR-503	-92.20	0.037
	miR-200b	94.52	0.048
	miR-133b	1731.74	0.014
VBD+IBS	miR-1825	-19012.5	0.019
	miR-1288	-745.74	0.045
	miR-593	-56.76	0.019
	let-7f-2#	5.77	0.034
	miR-512-3p	23.03	0.046
	miR-125a-3p	69.59	0.041
	miR-661	981.98	0.038
BOTH	miR-485-5p	**VBD:** -90.76	0.003
		VBD+IBS: -84.32	0.010
	miR-1294	**VBD:** -41.47	0.012
		VBD+IBS -60.67	0.014
	miR-520f	**VBD:** -33.67	0.003
		VBD+IBD: -15.24	0.035
	miR-520D-3p	**VBD:** 2486.77	0.021
		VBD+IBS: 204617	0.002

Figure 4.5 MicroRNA expression signatures are altered in women with VBD and VBD+IBS. Compared to HC, women with VBD exhibit significant downregulation of 7 and upregulation of 3 miRNAs **(A)**, while women with VBD+IBS exhibit significant downregulation of 6 and upregulation of 5 miRNAs **(B)**. Red dots represent downregulated miRNAs and blue dots represent upregulated miRNAs compared to HC. Four miRNAs are dysregulated in both VBD and VBD+IBS women. Dysregulated miRNAs are listed with fold change and FDR-adjusted p-values **(C)**. Abbreviations: false detection rate (FDR), healthy control (HC), irritable bowel syndrome (IBS), miRNA (microRNA), vestibulodynia (VBD).

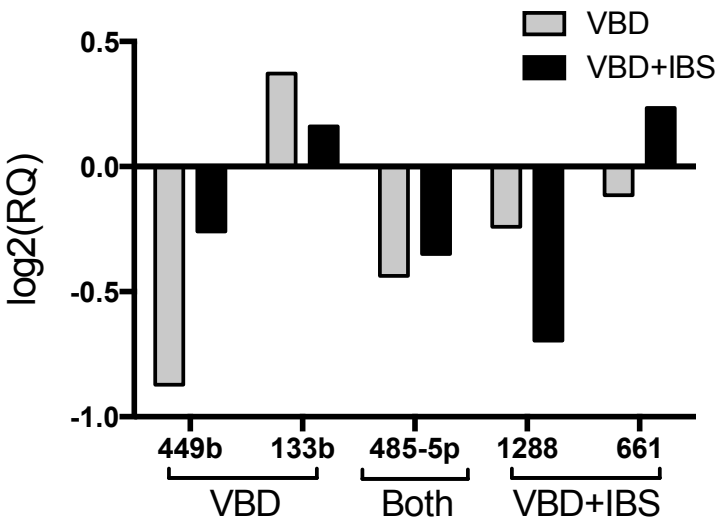


Figure 4.6 MicroRNA expression in VBD and VBD+IBS is validated by real-time PCR. Consistent with our miRNA array results, women with VBD (N=5) have decreased miR-449b and increased miR-133b whereas women with VBD+IBS (N=5) have decreased miR-1288 and increased miR-661 as compared to HC (N=5). Women in both groups have decreased miR-485-5p as compared to HC. Abbreviations: healthy control (HC), irritable bowel syndrome (IBS), vestibulodynia (VBD).

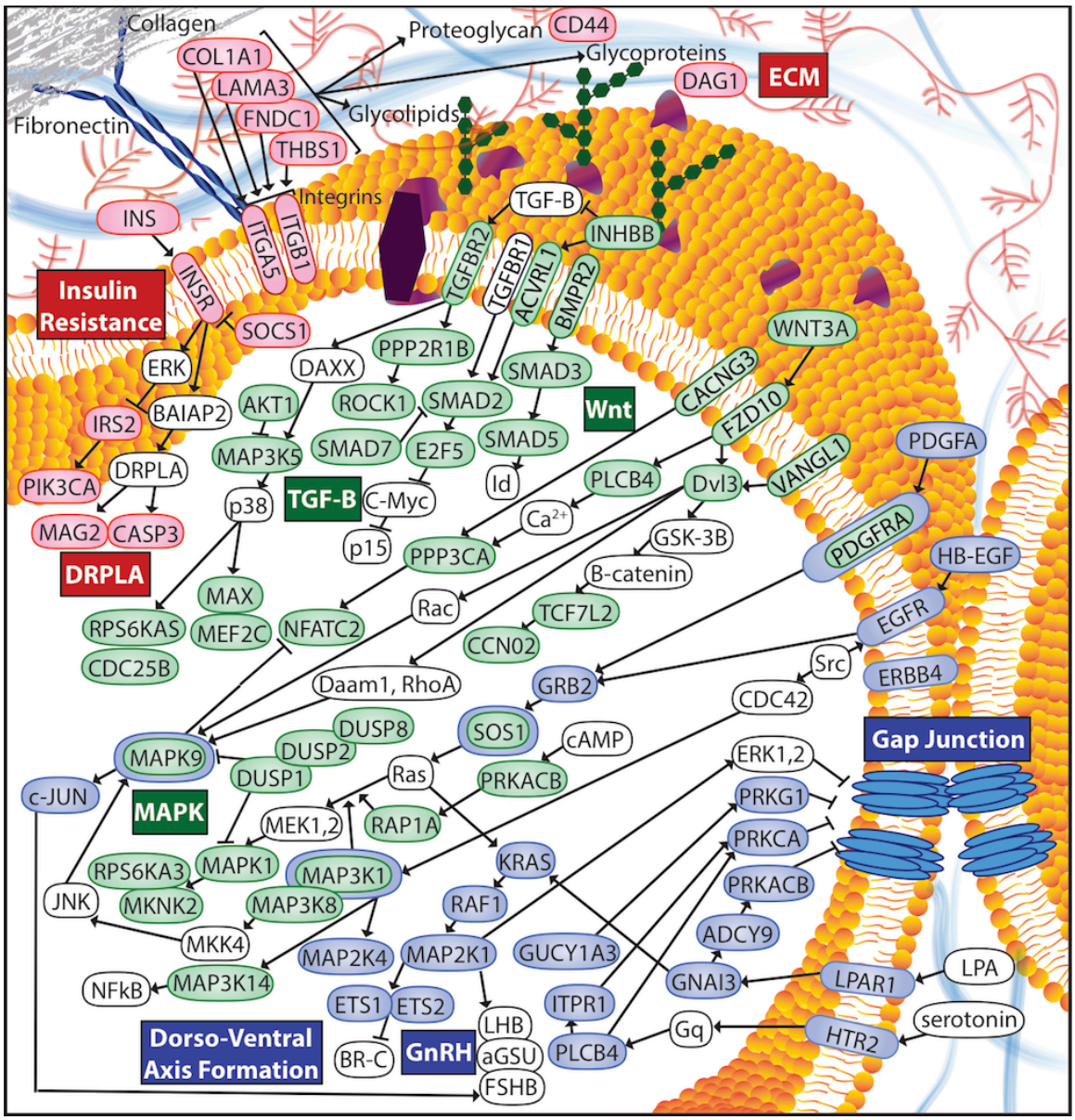


Figure 4.7 MicroRNA dysregulation affects multiple genes and pathways in women with VBD and VBD+IBS. miRNA dysregulation in women with VBD and VBD+IBS leads to dysregulation of genes involved in pain-relevant pathways including the TGFβ, MAPK, and Wnt signaling pathways (shown in green). Pathways that do not overlap between women with VBD and VBD+IBS represent separate pathways of vulnerability. Pathways unique to VBD (shown in blue) include the dorso-ventral axis formation, GnRH signaling, and gap junction pathways. Pathways unique to VBD+IBS (shown in red) include the ECM, DRPLA, and insulin resistance pathways. A considerable amount of interaction and communication is demonstrated between genes and across pathways. Abbreviations: healthy control (HC), irritable bowel syndrome (IBS), miRNA (microRNA), vestibulodynia (VBD). See Appendix 1 for all other abbreviations.

CHAPTER 5

SUMMARY

The studies presented in this dissertation aim to explore the roles of, and the relationship between, catechol-O-methyltransferase (COMT) and microRNAs (miRNAs) in idiopathic pain disorders (IPDs):

The first set of studies identifies a site of action for COMT-dependent pain. Chapter 2 utilizes a pharmacologic rat model to demonstrate that the initiation of persistent COMT-dependent pain is mediated *via* peripherally located β_2- and β_3ARs. Chapter 3 utilizes a genetic mouse model to demonstrate that established COMT-dependent pain is not mediated *via* peripherally located β_2- and β_3ARs. These exciting findings suggest that β_2- and β_3AR antagonist therapy may be effective, particularly for blocking the development of pain in individuals experiencing prolonged elevations in catecholamines associated with environmental stressors or epigenetic events. Still, more research is required to identify the cell types and elucidate the pathways involved in the onset and maintenance of COMT-dependent pain and to discover an effective treatment for established COMT-dependent pain.

The next set of studies identifies miRNA dysregulation and downstream pathways affected by persistent idiopathic pain. Specifically, Chapter 4 demonstrates that miRNA dysregulation is also present in clinical IPDs and that miRNA expression profiles can differentiate between IPD subtypes with distinct phenotypic characteristics. These exciting results suggest that miRNAs may possess utility as biomarkers, both to diagnose and to differentiate between subtypes of COMT-dependent pain and IPDs. They also suggest that

miRNAs may represent treatment targets for persistent idiopathic pain. Ongoing studies by our group are currently focused on linking the COMT story with miRNAs by observing miRNA expression in the pharmacologic rat model of COMT-dependent pain. The preliminary results for this work can be found in Appendix 1. It is important to note that these miRNA studies are a narrow view of an extremely complex regulatory system. Extensive research is necessary to elucidate the role of individual miRNAs in IPDs and to identify downstream proteins and pathways affected by miRNA dysregulation, so that more effective therapeutics can be developed.

Idiopathic pain is a major healthcare problem, difficult to prevent and ineffectively treated due to its unclear etiology and heterogeneous presentation. The diverse array of molecular, cellular, physiological, and psychological IPD attributes makes understanding the complexities of persistent idiopathic pain an ambitious task. Success will require a multi-disciplinary effort that forces specialists to collaborate and think outside the box. The studies presented in this dissertation link the known fact that COMT is associated with IPDs with the idea that miRNAs may play a role in IPDs. The results of these studies work to unravel some of the complexities of persistent idiopathic pain, identifying novel mechanisms underlying IPDs as well as possible avenues that may lead to effective therapeutics.

APPENDIX 1:
ONGOING RODENT STUDIES EXPLORING THE RELATIONSHIP BETWEEN MICRORNA EXPRESSION AND CATECHOL-O-METHYLTRANSFERASE-DEPENDENT PAIN

Introduction

Though the etiologies underlying idiopathic pain disorders (IPDs) are not well understood, emerging evidence indicates a role for catechol-O-methyltransferase (COMT), as well as β-adrenergic receptors (βARs). Idiopathic pain has been previously associated with both diminished activity of COMT,[127,128] an enzyme responsible for catabolizing catecholamines, as well as increased levels of circulating catecholamines.[131-133] Furthermore, functional variants in the COMT gene that reduce COMT activity[128,196,197] are associated with increased susceptibility to IPDs[141,145,146,198-200] as well as enhanced experimental pain[141,201] and impaired response to treatment.[139,202] Previous work performed by our group utilized a pharmacologic rat model to demonstrate that sustained administration of the COMT inhibitor OR486 causes enhanced mechanical and thermal hypersensitivity. This hypersensitivity, however, can be blocked by sustained peripheral administration of β_2- and β_3AR antagonists, demonstrating that the initiation of COMT-dependent pain is mediated *via* peripherally located β_2- and β_3ARs (Chapter 2).[274] Recent work has demonstrated that β_2- and β_3ARs drive COMT-dependent pain by increasing the production of inflammatory mediators such as nitric oxide and pro-inflammatory cytokines.[396] More research is necessary, however, to understand the mechanisms downstream of βAR activation that lead to alterations in expression of pain-relevant molecules.

Emerging evidence suggests that epigenetic mechanisms can silence expression of genes that are pro- or anti-nociceptive, thus altering pain pathways.[86,87] MicroRNAs (miRNAs), for example, are a type of epigenetic regulation that could affect pain-relevant processes.[67] MiRNAs are small, non-coding RNAs that can regulate or control gene expression by inhibiting protein translation or degrading downstream target mRNAs.[297]

Dysregulation of miRNAs can cause subsequent dysregulation of downstream pathways and processes, often resulting in disease. MiRNA dysregulation has been observed in various diseases including, but not limited to, multiple sclerosis,[94] peripheral artery disease,[95] major depressive disorder,[96] cardiovascular disease,[97] liver injury,[98] and cancer.[99] Circulating miRNAs are ideal biomarkers because they are stable in serum and plasma.[101] Understanding miRNA expression profiles for persistent pain would be extremely beneficial to the field, as it would help us to identify pathways of interest for treatment. Additionally, identifying miRNA biomarkers of persistent pain would be groundbreaking, as there are currently no objective diagnostic tests that can assess an individual's severity and type of pain.[397]

MiRNAs play an important role in many pain-relevant processes. They are necessary for development and normal function of the immune system. Abnormal immune system function can lead to overproduction of inflammatory mediators (*e.g.,* cytokines), contributing to chronic pain conditions.[102,103] Several miRNAs have been identified as regulators or modulators of pain-relevant pathways such as the nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κB),[109,110] mitogen-activated protein kinase (MAPK),[398] and janus kinase and signal transducer and activator of transcription (JAK-STAT)[399,400] pathways, as well as of catecholamine[184] and βAR signaling pathways.[180,182,401] Rodent studies have linked miRNA expression to both inflammatory[111,112] and neuropathic[112,114,115] pain, as well as to nociceptor excitability and pain thresholds.[116] Intrathecal injections of pain-regulating miRNAs have been shown to prevent and alleviate pain in rodents.[117,118] In humans, miRNA dysregulation has been linked to pain-relevant conditions such as osteoarthritis (OA),[120] complex regional pain syndrome (CRPS), [121] chronic visceral pain,[402] fibromyalgia,[122] bladder pain syndrome (BPS),[123] migraines,[124] vestibulodynia (VBD),[395] and irritable bowel syndrome (IBS).[395] MiRNA expression in COMT-dependent pain, however, has yet to be studied.

Identifying miRNA expression patterns and affected downstream pathways will help us to better understand epigenetic regulation of COMT-dependent pain as well as to elucidate new therapeutic targets. We hypothesize that aberrant catecholamine signaling and overstimulation of βARs in COMT-dependent pain is associated with miRNA dysregulation. To test this hypothesis, we examined miRNA expression profiles in rats receiving COMT inhibitor OR486 alongside vehicle or βAR antagonists. Though the results are preliminary at this time, miRNA dysregulation was observed in both groups. MiRNA dysregulation overlapped slightly between groups, suggesting that not all OR486-induced miRNA dysregulation is dependent on βAR signaling. We also found that OR486 administration alongside vehicle, but not βAR antagonists, causes dysregulation of circulating miRNAs responsible for regulating pain-relevant pathways. Ongoing investigation of these data will help to confirm these results and elucidate the role of miRNAs in COMT-dependent pain.

Materials and Methods

Animals

Adult female Sprague-Dawley rats (N=20) were bred in house, weighed between 200 and 400g, and had *ad libitum* access to standard laboratory chow and water. All animal procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of North Carolina at Chapel Hill (UNC). Though rodent models of pain only partially correlate with human conditions, rats were chosen for these experiments because an extensive body of literature exists regarding nociceptive pathways and behavior in this species, and because rat pain behavior assays are readily available and well characterized.[7,205,206] Females were chosen for the present study because despite the fact that persistent pain conditions are more prevalent in females than in males,[192] basic science experiments in all fields of study, including pain, utilize male animals almost exclusively.[403]

General Experimental Conditions

Previous work has characterized a rat model for the initiation of persistent COMT-dependent pain, demonstrating that persistent administration of the COMT inhibitor OR486 leads to enhanced mechanical and thermal pain. This pain can be blocked by peripheral administration of the selective β_2AR antagonist ICI-118,511 (ICI) or the selective β_3AR antagonist SR59230A (SR) (See Chapter 2).[274] Here, we aimed to identify miRNAs that are dysregulated following sustained COMT inhibition and to determine the ability of β_2- and β_sAR antagonists to prevent COMT-dependent abnormalities in miRNA expression. Separate groups of rats received persistent subcutaneous (s.c.) administration of OR486 or Vehicle (Veh) for a 2-week period *via* a 2002 Alzet osmotic mini-pump (Durect Corporation, Cupertino, CA) together with peripheral administration of either ICI-SR or Veh *via* a second osmotic mini-pump. Pumps were implanted surgically on Day 0 to deliver Veh/Veh, OR486/Veh, Veh/SR-ICI and OR486/SR-ICI (N=5 per group) and whole blood collected on Day 14 of the experimental paradigm.

Drug Preparation

OR486 (Tocris, Ellisville, MO), ICI (Tocris, Ellisville, MO), and SR (Tocris, Ellisville, MO) were each dissolved in a 5:3:2 ratio of dimethylsulfoxide (DMSO), 0.9%saline, and ethanol.[147] Drug solutions were injected into pumps, which were placed in 15mL conical tubes containing sterile 0.9% saline and primed overnight in a dry heat bath (Lab Armor, Cornelius, OR) at 37 degrees Celsius. Subcutaneous delivery of OR486 was at a constant rate of 15mg/kg/day for the two-week period. Peripheral delivery of propranolol ICI-118,511 was at 1.5mg/kg/day and SR59230A was at 1.67mg/kg/day, also for the two-week period.

Surgical Procedures

For all surgical procedures, rats were anesthetized by isoflurane inhalation (5% induction, 1.5-5% maintenance). Incision sites were shaved and disinfected with ethanol and betadine. Sterile technique was employed throughout the duration of all procedures

according to IACUC requirements. Stainless steel wound clips (Braintree Scientific, Braintree, MA) were used to close the wounds. A small incision was made over the shoulder blades of the rat. Hemostats were used to create a small subcutaneous pocket, in which both pumps were placed (the first for OR486 or Veh and the second for ICI-SR or Veh).

miRNA Profiling

Whole blood was collected from rats in PAXgene Blood RNA tubes (BD Biosciences, Franklin Lakes, NJ, USA). Tubes were stored at room temperature for 48 hours, as recommended by the vendor, and then used immediately for RNA isolation. RNA was isolated from blood using the PAXgene blood miRNA kit (Qiagen, Germantown, MD, USA). The quantity and quality of the RNA was measured using a NanoDrop 1000 (NanoDrop Technologies, Wilmington, DE, USA); and the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA), respectively. RNase-free water was used to normalize all samples to ~250ng/uL. RNA Integrity Number (RIN) values can be found in Table 4.1. RNA samples were then shipped to the Next Generation Sequencing and Bioinformatics Core at the University of Texas Health Science Center at San Antonio for small RNA sequencing using the Illumina HiSeq 2000 (Illumina Inc., San Diego, CA, USA) with the TriLink Small RNA Library Prep Kit (three biological replicates; Trilink, San Diego, CA, USA).

Statistical Analysis

MiRNA expression was quantified using a previously described small RNA-sequencing Read Mapping Pipeline.[404] This pipeline, which allows for identification of established miRNAs as well as potential novel miRNAs, utilizes the Bowtie[405] and SHRiMP[406] software packages. This pipeline has been used in previous studies to predict candidate miRNAs that may act as regulatory hubs for a particular disease. All miRNAs with $p<0.06$ are reported in the present study.

miRNA Pathway Analysis

MiRNA expression profiles in blood may inform us of both peripheral and central pain processes affected by persistent OR486-induced COMT-dependent pain. Samples of circulating blood provide a rich source of pain-relevant molecules, including neurochemicals released by sympathetic nerve terminals and inflammatory mediators released by circulating immune cells.[315] Furthermore, studies have shown that whole blood shares significant gene[316] and miRNA[317] expression similarities with CNS tissues. To determine the genes and pathways affected by miRNA expression in our rat model of COMT-dependent pain, we performed pathway analysis using the *in silico* DIANA miRPath v2.0 analysis software:[320] (http://diana.imis.athena-innovation.gr/DianaTools; False Discovery Rate Correction: ON; Conservative Stats Setting: ON). The analysis generated a list of genes affected by changes in miRNA expression in each group and organized them into Kyoto Encyclopedia of Genes and Genomes (Kegg) pathways. For each pathway, a p-value that accounts for all affected genes was also generated. Information on miR-451-5p, miR-17-1-5p, miR-17-1-3p, and miR-17-2-5p is extremely limited, therefore they are not included in the pathway analysis.

Preliminary Results and Discussion

As miRNAs are key regulators of processes related to pain, psychological variables, and inflammatory responses, and because they have been linked to IPDs, we explored their expression in rats receiving the COMT inhibitor OR486 alongside Vehicle or ICI-SR. Thus far, we have completed miRNA isolation, sequencing, and preliminary analysis of the whole blood samples. RNA concentrations and RNA integrity numbers (RINs) can be found in Table A1.1. The length distribution of small RNA reads (max=50) ranges between 14 to 42 nucleotides for these samples, with the most prevalent lengths falling between 18 to 24 and 29 to 31 nucleotides. The most prevalent length observed for small RNAs in these samples was 22 nucleotides (26.63% of reads; Figure A1.1). Our results are consistent with those of

previous studies that have recognized the most common length for miRNAs to be 21 to 22 nucleotides.[407,408] Our results are also consistent with that of previous length distribution studies, which have shown similar length distribution curves for small RNAs in both human and mouse samples.[409] The increased prevalence of small RNAs in the 29 to 31 nucleotide range in our samples may suggest the presence of stress-induced tRNA-derived RNAs, which are generally 30 to 35 nucleotides in length.[410]

Preliminary sequencing analysis has demonstrated that the most statistically relevant miRNAs in the pharmacologic rat model of COMT-dependent pain (OR186/Veh) as compared to the control group include miR-133a-3p, miR-451-5p, miR-93-5p, miR-872-5p, miR-17-1-3p, miR-30e-5p, miR-17-1-5p, miR-181d-5p, miR-17-2-5p, and miR-652-3p (Table A1.2). Though these miRNAs do not overlap with those found to be dysregulated in IPD patient populations (Chapter 4),[395] predicted pathways affected by OR486/Veh miRNA dyrsregulation include the mitogen-activated protein kinase (MAPK) and the tumor growth factor beta (TGF-β) pathways (Table A1.3). Each of these pathways were also pathways of interest for VBD and VBD+IBS patients (Chapter 4). These data parallel those of various previous studies, linking the MAPK and TGF-β pathways to pain, inflammation, and IPDs.[395]

We next observed circulating miRNA expression profiles of rats receiving OR486 alongside coadministration of ICI and SR, which are βAR antagonists known to block the development of OR486-induced pain (Chapter 2). The most statistically relevant miRNAs in the OR486/ICI-SR group include miR-182, miR-106b-3p, miR-150-5p, miR-99b, miR-30e-5p, miR-16-5p, miR-342-3p, miR-93-5p, miR-93-5p, miR-181d-5p, let-7d-5p, and let-7b-5p (Table A1.2). The miRNA expression profile of this group as compared to the Veh/Veh group differed from that of the OR486/Veh group, demonstrating that ICI-SR administration may help to alleviate OR486-induced pain *via* alteration of miRNA expression. Three of the miRNAs overlapped with those of the OR486/Veh group (miR-93-5p, miR-30e-5p, miR-181d-5p), demonstrating that some OR486-induced miRNA alterations occur independent of

βAR-signaling. Unlike the OR486/Veh group, miRNA dysregulation in the OR486/ICI-SR group is not predicted to affect the pain-relevant MAPK and TGF-β pathways (Table A1.4). These results suggest that βAR antagonists may block the development of COMT-dependent pain by altering miRNA expression and thereby preventing the activation of pain-relevant pathways (e.g., MAPK, TGF-β). Of note, the number of predicted genes affected per pathway is very small in the OR486/ICI-SR group (Table A1.4) compared to that of the OR486/Veh group (Table A1.3). Further, there are far less total predicted pathways affected (3 in OR486/ICI-SR vs 29 in OR486/Veh), suggesting that βAR antagonists are protective of miRNA expression alterations and downstream pathway dysregulation initiated by OR486 administration. Only one miRNA was dysregulated in the Veh/ICI-SR group as compared to controls (miR-133a-3p; FC=0.321; $p<0.033$). This miRNA is also downregulated in the OR486/Veh group, suggesting it may be altered in response to the vehicle used for dilution of OR486 and antagonists.

Collectively, these data demonstrate that βAR antagonist treatment may alleviate OR486-induced pain by blocking the development of miRNA and pathway dysregulation downstream of βAR-signaling. Together with Chapter 4, these data suggest an important role for the MAPK and TGF-β pathways in IPDs as well as COMT-dependent pain. These pathways, along with the specific miRNAs identified in these studies, may represent target treatments for patients who suffer from persistent pain. Further investigation will help to elucidate the role of each individual miRNA, gene, and pathway identified in this preliminary analysis, allowing us to develop more efficient treatment plans for those who suffer from IPDs.

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Footnotes

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Table A1.1 RNA Concentrations and RIN Values

Rat ID Number	Group	Original Concentration	RIN
1	OR486/Veh	302 ng/uL	7.4
2	OR486/ICI-SR	482 ng/uL	6.8
3	Veh/Veh	351 ng/uL	4.1
4	Veh/ICI-SR	460 ng/uL	6.7
5	OR486/Veh	388 ng/uL	7.3
6	OR486/ICI-SR	449 ng/uL	6.9
7	Veh/Veh	1492 ng/uL	6.1
8	Veh/ICI-SR	1618 ng/uL	6.2
9	OR486/Veh	767 ng/uL	6.1
10	OR486/ICI-SR	788 ng/uL	6.3
11	Veh/Veh	256 ng/uL	7.8
12	Veh/ICI-SR	1797 ng/uL	6.3
13	OR486/Veh	862 ng/uL	5.6
14	OR486/ICI-SR	407 ng/uL	6.7
15	Veh/Veh	318 ng/uL	7.3
16	Veh/ICI-SR	1350 ng/uL	5.2
17	OR486/Veh	811 ng/uL	3.5
18	OR486/ICI-SR	944 ng/uL	5.6
19	Veh/Veh	745 ng/uL	6.9
20	Veh/ICI-SR	905 ng/uL	5.6

Abbreviations: ICI118,511 (ICI), RNA Integrity Number (RIN), SR59230A (SR), Vehicle (Veh).

Table A1.2 MicroRNA Dysregulation in a Rodent Model of OR486-Induced COMT-Dependent Pain

	miRNA	Fold Change	p-value
OR486/Veh	miR-133a-3p	0.156	0.032
	miR-451-5p	0.540	0.025
	miR-93-5p	0.500	0.036
	miR-872-5p	0.494	0.036
	miR-17-1-3p	0.589	0.038
	miR-30e-5p	0.539	0.051
	miR-17-1-5p	0.454	0.054
	miR-181d-5p	0.631	0.054
	miR-17-2-5p	0.458	0.059
	miR-652-3p	0.693	0.059
OR486/ICI-SR	miR-182	0.788	0.008
	miR-106b-3p	0.614	0.024
	miR-150-5p	2.466	0.027
	miR-99b-5p	1.512	0.029
	miR-30e-5p	0.581	0.031
	miR-16-5p	0.394	0.032
	miR-342-3p	2.026	0.038
	miR-93-5p	0.579	0.038
	miR-181d-5p	0.728	0.042
	let-7d-5p	0.680	0.044
	let-7b-5p	0.744	0.057

MicroRNAs with differential expression as compared to the Veh/Veh group ($p<0.06$) are shown here with corresponding fold change and p-values. Upregulated miRNAs are shown in blue and downregulated miRNAs are shown in red. Overlapping miRNAs are shown in **bold**. Abbreviations: Vehicle (Veh), ICI-118,551 (ICI), microRNA (miRNA), SR59230A (SR).

Table A1.3 Predicted miRNA Pathway Dysregulation in Rats Receiving OR486/Veh

Pathway	p-value	# genes
Axon guidance	<0.0001	41
MAPK signaling pathway	<0.0001	31
Osteoclast differentiation	<0.0001	26
Regulation of actin cytoskeleton	<0.0001	29
Transcriptional misregulation in cancer	<0.0001	22
Endocytosis	0.0001	18
Mucin type-O-Glycan biosynthesis	0.0001	4
Long-term potentiation	0.0001	15
Prostate cancer	0.0001	14
Circadian rhythm	0.0002	10
ErbB signaling pathway	0.0002	20
T cell receptor signaling pathway	0.0003	19
Chronic myeloid leukemia	0.0003	14
Glutamategic synapse	0.0006	19
VEGF signaling pathway	0.0010	11
Neurotrophin signaling pathway	0.0026	21
Acute myeloid leukemia	0.0052	10
TGF-beta signaling pathway	0.0059	14
Glioma	0.0075	11
Calcium signaling pathway	0.0085	22

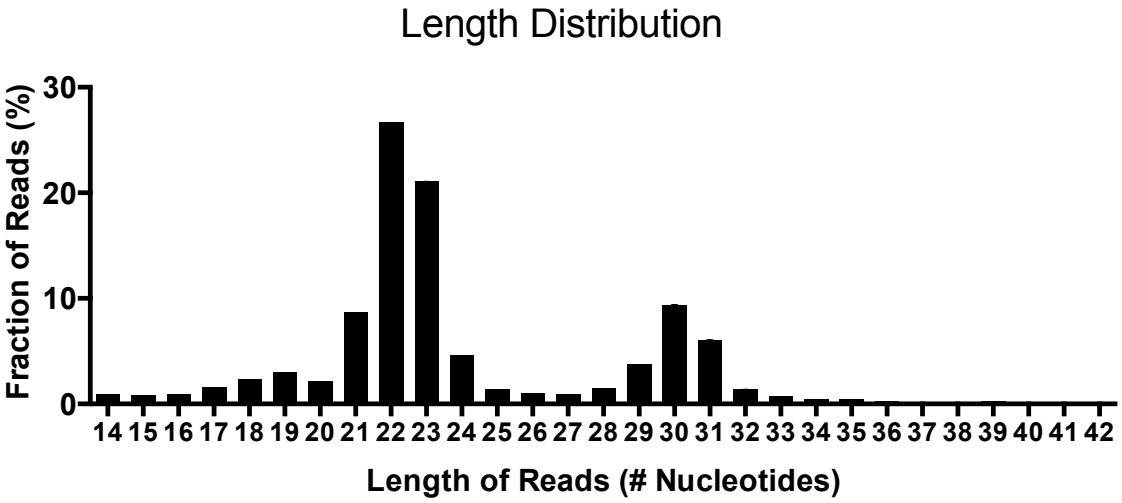
The top 20 pathways, as determined by the DIANA miRPath v2.0 Analysis Software, affected by miRNA dysregulation in rats receiving OR486 alongside Veh, as compared to the Veh/Veh group. Pathways in bold overlap with that of the OR486/ICI-SR Group. Abbreviations: ICI-118,551 (ICI), mitogen-activated protein kinase (MAPK), SR59230A (SR), tumor growth factor beta (TGF-beta), vascular endothelial growth factor (VEGF)

Table A1.4 Predicted miRNA Pathway Dysregulation in Rats Receiving OR486/ICI-SR

Pathway	p-value	# genes
Pathways in cancer	0.0001	4
Renal cell carcinoma	0.0174	2
Glioma	0.0316	2
Axon Guidance	0.0432	2

All pathways ($p<0.05$), as determined by the DIANA miRPath v2.0 Analysis Software, affected by miRNA dysregulation in rats receiving OR486 alongside the β_2- and β_3AR antagonists ICI and SR, as compared to the Veh/Veh group. Pathways in bold overlap with that of the OR486/Veh Group. Abbreviations: hypoxia-inducible factor 1 (HIF-1), ICI-118,551 (ICI), mitogen-activated protein kinase (MAPK), SR59230A (SR), vascular endothelial growth factor (VEGF).

Figure A1.1 Small RNA Reads Length Distribution



APPENDIX 2: ABBREVIATIONS FOR FIGURE 5.7

Abbreviations: insulin (INS), insulin receptor (INSR), extracellular signal-regulated kinases (ERK), insulin receptor substrate 2 (IRS2), phosphatidylinositol-4,5-Bisphosphate 3-Kinase, Catalytic Subunit Alpha (PIK3CA), brain-specific angiogenesis inhibitor 1-associated protein 2 (BAIAP2), dentatorubral-pallidoluysian atrophy (DRPLA), caspase 3 (CASP3), suppressor of cytokine signaling 1 (SOCS), alpha-1 type I collagen (COL1a1), laminin alpha-3 (LAMA3), fibronectin type III domain containing 1 (FNDC1), thrombospondin 1 (THBS1), integrin alpha-5 (ITGA5), integrin beta-1 (ITGB1), dystroglycan 1 (DAG1), extracellular matrix (ECM), transforming growth factor beta (TGF-B), TGF-B receptor 2 (TGFBR2), TGF-B receptor 1 (TGFBR1), death domain-associated protein (DAXX), RAC-alpha serine/threonine-protein kinase (AKT1), mitogen-activated protein kinase (MAPK), ribosomal protein S6 kinase 90kDa polypeptide 3 (RPS6KA3), cell division cycle 25B (CDC25B), MYC-associated factor X (MAX), myocyte enhancer factor 2C (MEF2C), protein phosphatase 2 regulatory subunit A beta (PPP2R1B), Rho-associated coiled-coil containing protein kinase 1 (ROCK1), E2F transcription factor 5 p130-binding (E2F5), cyclin-dependent kinase 4 inhibitor B (p15), inhibin beta B (INHBB), activin A receptor type II-like 1 (ACVRL1), wingless-type MMTV integration site family member 3A (WNT3A), frizzled class receptor 10 (FZD10), phospholipase C beta 4 (PLCB4), protein phosphatase 3 catalytic subunit alpha isozyme (PPP3CA), nuclear factor of activated T cells cytoplasmic calcineurin-dependent 2 (NFATC2), jun proto-oncogene (c-JUN), disheveled segment polarity protein 3 (DvI3), glycogen synthase kinase 3 beta (GSK-3B), beta-catenin (B-catenin), transcription factor 7-like 2 (TCF7L2), disheveled-associated activator of morphogenesis 1 (Daam1), ras homolog family member A (RhoA), VANGL Planar Cell Polarity Protein 1 (VANGL1), dual-specificity protein phosphatase (DUSP), platelet-derived growth factor alpha polypeptide (PDGFA), PDGF receptor alpha polypeptide (PDGFRA), growth factor receptor-bound protein 2 (GRB2), son of sevenless homolog 1 (SOS1), methyl ethyl ketone (MEK), MAPK interacting serine/threonine kinase 2 (MKNK2), cyclic adenosine monophosphate (cAMP), protein kinase cAMP-dependent catalytic beta (PRKACB), member of ras oncogene family (RAP1A), MAPK kinase 4 (MKK4), c-Jun N-terminal kinase (JNK), nuclear factor kappa beta (NFkB), Kirsten rat sarcoma viral oncogene homolog (KRAS), proto-oncogene c-RAF (RAF1), v-ets erythroblastosis virus E26 oncogene homolog (ETS), broad-complex (BR-C), gonadotropin-releasing hormone (GnRH), luteinizing hormone beta polypeptide (LHB), alpha gonadotropin (aGSU), follicle-stimulating hormone beta subunit (FSHB), heparin-binding EGF-like growth factor (HB-EGF), epidermal growth factor receptor (EGFR), proto-oncogene c-Src (Src), cell-division control protein 42 homolog (CDC42), v-erb-b2 avian erythroblastic leukemia viral oncogene homolog 4 (ERBB4), protein kinase cGMP-dependent type I (PRKG1), protein kinase C alpha (PRKCA), PRK cAMP-dependent catalytic beta (PRKACB), adenylate cyclase 1 (ADCY1), G protein alpha inhibiting activity polypeptide 3 (GNAI3), guanylate cyclase 1 alpha 3 (GUCY1A3), inositol 1,4,5-trisphosphate receptor type 1 (ITPR1), phospholipase C beta 4 (PLCB4), lysophosphatidic acid receptor 1 (LPAR1), lipoprotein (LPA), serotonin receptor (HTR2).

REFERENCES

1. (IASP) TIAftSoP. IASP Taxonomy. Available at: http://www.iasp-pain.org/Taxonomy - Pain Accessed: January 2016

2. Peddareddygari LR, Oberoi K, Grewal RP: Congenital insensitivity to pain: a case report and review of the literature. Case Rep Neurol Med 2014; 2014: 141953

3. American Pain Foundation. Pain in the Workplace: A 10-year Update. Available at: http://www.painfoundation.org/page.asp?file=Library/Index.htm Accessed September 2008

4. Max MB: How to move pain and symptom research from the margin to the mainstream. J Pain 2003; 4: 355-60

5. Medicine TAAoP. AAPM Facts and Figures on Pain. Available at: http://www.painmed.org/patientcenter/facts_on_pain.aspx - chronic Accessed: January 2016

6. Cohen SP, Mao J: Neuropathic pain: mechanisms and their clinical implications. BMJ 2014; 348: f7656

7. Berge OG: Predictive validity of behavioural animal models for chronic pain. Br J Pharmacol 2011; 164: 1195-206

8. Bingefors K, Isacson D: Epidemiology, co-morbidity, and impact on health-related quality of life of self-reported headache and musculoskeletal pain--a gender perspective. Eur J Pain 2004; 8: 435-50

9. Malec-Milewska MB, Tarnowski W, Ciesielski AE, Michalik E, Guc MR, Jastrzebski JA: Prospective evaluation of pain control and quality of life in patients with chronic pancreatitis following bilateral thoracoscopic splanchnicectomy. Surg Endosc 2013; 27: 3639-45

10. Almakadma YS, Simpson K: Opioid therapy in non-cancer chronic pain patients: Trends and efficacy in different types of pain, patients age and gender. Saudi J Anaesth 2013; 7: 291-5

11. Diatchenko L, Fillingim RB, Smith SB, Maixner W: The phenotypic and genetic signatures of common musculoskeletal pain conditions. Nat Rev Rheumatol 2013; 9: 340-50

12. Harlow BL, Vazquez G, MacLehose RF, Erickson DJ, Oakes JM, Duval SJ: Self-reported vulvar pain characteristics and their association with clinically confirmed vestibulodynia. J Womens Health (Larchmt) 2009; 18: 1333-40

13. Ciszek BP, Khan AA, Dang H, Slade GD, Smith S, Bair E, Maixner W, Zolnoun D, Nackley AG: MicroRNA expression profiles differentiate chronic pain condition subtypes. Transl Res 2015; 166: 706-720 e11

14. Ohrbach R, Fillingim RB, Mulkey F, Gonzalez Y, Gordon S, Gremillion H, Lim PF, Ribeiro-Dasilva M, Greenspan JD, Knott C, Maixner W, Slade G: Clinical findings and pain

symptoms as potential risk factors for chronic TMD: descriptive data and empirically identified domains from the OPPERA case-control study. J Pain 2011; 12: T27-45

15. Kitaj MB, Klink M: Pain thresholds in daily transformed migraine versus episodic migraine headache patients. Headache 2005; 45: 992-8

16. Roditi D, Robinson ME: The role of psychological interventions in the management of patients with chronic pain. Psychol Res Behav Manag 2011; 4: 41-9

17. Nekovarova T, Yamamotova A, Vales K, Stuchlik A, Fricova J, Rokyta R: Common mechanisms of pain and depression: are antidepressants also analgesics? Front Behav Neurosci 2014; 8: 99

18. Hulsebusch J, Hasenbring MI, Rusu AC: Understanding Pain and Depression in Back Pain: the Role of Catastrophizing, Help-/Hopelessness, and Thought Suppression as Potential Mediators. Int J Behav Med 2015

19. Minen MT, Begasse De Dhaem O, Kroon Van Diest A, Powers S, Schwedt TJ, Lipton R, Silbersweig D: Migraine and its psychiatric comorbidities. J Neurol Neurosurg Psychiatry 2016

20. Borchers AT, Gershwin ME: Fibromyalgia: A Critical and Comprehensive Review. Clin Rev Allergy Immunol 2015; 49: 100-51

21. Lundqvist EN, Bergdahl J: Vestibulodynia (former vulvar vestibulitis): personality in affected women. J Psychosom Obstet Gynaecol 2005; 26: 251-6

22. Kibune Nagasako C, Garcia Montes C, Silva Lorena SL, Mesquita MA: Irritable bowel syndrome subtypes: Clinical and psychological features, body mass index and comorbidities. Rev Esp Enferm Dig 2015; 108

23. Lynch ME, Watson CP: The pharmacotherapy of chronic pain: a review. Pain Res Manag 2006; 11: 11-38

24. Navratilova E, Morimura K, Xie JY, Atcherley CW, Ossipov MH, Porreca F: Positive emotions and brain reward circuits in chronic pain. J Comp Neurol 2016

25. Asmundson GJ, Katz J: Understanding the co-occurrence of anxiety disorders and chronic pain: state-of-the-art. Depress Anxiety 2009; 26: 888-901

26. Simons LE, Elman I, Borsook D: Psychological processing in chronic pain: a neural systems approach. Neurosci Biobehav Rev 2014; 39: 61-78

27. Trivedi MH: The link between depression and physical symptoms. Prim Care Companion J Clin Psychiatry 2004; 6: 12-6

28. Peters ML: Emotional and Cognitive Influences on Pain Experience. Mod Trends Pharmacopsychiatri 2015; 30: 138-52

29. Sharp TJ, Harvey AG: Chronic pain and posttraumatic stress disorder: mutual maintenance? Clin Psychol Rev 2001; 21: 857-77

30. Parkin J, Cohen B: An overview of the immune system. Lancet 2001; 357: 1777-89

31. Zhang JM, An J: Cytokines, inflammation, and pain. Int Anesthesiol Clin 2007; 45: 27-37

32. Slade GD, Conrad MS, Diatchenko L, Rashid NU, Zhong S, Smith S, Rhodes J, Medvedev A, Makarov S, Maixner W, Nackley AG: Cytokine biomarkers and chronic pain: association of genes, transcription, and circulating proteins with temporomandibular disorders and widespread palpation tenderness. Pain 2011; 152: 2802-12

33. Rodriguez-Pinto I, Agmon-Levin N, Howard A, Shoenfeld Y: Fibromyalgia and cytokines. Immunol Lett 2014; 161: 200-3

34. Uceyler N, Sommer C: Cytokine regulation in animal models of neuropathic pain and in human diseases. Neurosci Lett 2008; 437: 194-8

35. Watkins LR, Maier SF: Immune regulation of central nervous system functions: from sickness responses to pathological pain. J Intern Med 2005; 257: 139-55

36. Kidd BL, Urban LA: Mechanisms of inflammatory pain. Br J Anaesth 2001; 87: 3-11

37. Latremoliere A, Woolf CJ: Central sensitization: a generator of pain hypersensitivity by central neural plasticity. J Pain 2009; 10: 895-926

38. Schafers M, Svensson CI, Sommer C, Sorkin LS: Tumor necrosis factor-alpha induces mechanical allodynia after spinal nerve ligation by activation of p38 MAPK in primary sensory neurons. J Neurosci 2003; 23: 2517-21

39. Zhang JM, Li H, Liu B, Brull SJ: Acute topical application of tumor necrosis factor alpha evokes protein kinase A-dependent responses in rat sensory neurons. J Neurophysiol 2002; 88: 1387-92

40. Woolf CJ: Central sensitization: implications for the diagnosis and treatment of pain. Pain 2011; 152: S2-15

41. Tajti J, Vecsei L: [The mechanism of peripheral and central sensitization in migraine. A literature review]. Neuropsychopharmacol Hung 2009; 11: 15-21

42. Nielsen LA, Henriksson KG: Pathophysiological mechanisms in chronic musculoskeletal pain (fibromyalgia): the role of central and peripheral sensitization and pain disinhibition. Best Pract Res Clin Rheumatol 2007; 21: 465-80

43. Park JW, Clark GT, Kim YK, Chung JW: Analysis of thermal pain sensitivity and psychological profiles in different subgroups of TMD patients. Int J Oral Maxillofac Surg 2010; 39: 968-74

44. Gibson SJ, Littlejohn GO, Gorman MM, Helme RD, Granges G: Altered heat pain thresholds and cerebral event-related potentials following painful CO2 laser stimulation in subjects with fibromyalgia syndrome. Pain 1994; 58: 185-93

45. Curatolo M, Petersen-Felix S, Arendt-Nielsen L, Giani C, Zbinden AM, Radanov BP: Central hypersensitivity in chronic pain after whiplash injury. Clin J Pain 2001; 17: 306-15

46. Jensen R: Mechanisms of spontaneous tension-type headaches: an analysis of tenderness, pain thresholds and EMG. Pain 1996; 64: 251-6

47. Moyal-Barracco M, Lynch PJ: 2003 ISSVD terminology and classification of vulvodynia: a historical perspective. J Reprod Med 2004; 49: 772-7

48. Edwards L: New concepts in vulvodynia. Am J Obstet Gynecol 2003; 189: S24-30

49. Bachmann GA, Rosen R, Pinn VW, Utian WH, Ayers C, Basson R, Binik YM, Brown C, Foster DC, Gibbons JM, Jr., Goldstein I, Graziottin A, Haefner HK, Harlow BL, Spadt SK, Leiblum SR, Masheb RM, Reed BD, Sobel JD, Veasley C, Wesselmann U, Witkin SS: Vulvodynia: a state-of-the-art consensus on definitions, diagnosis and management. J Reprod Med 2006; 51: 447-56

50. Groysman V: Vulvodynia: new concepts and review of the literature. Dermatol Clin 2010; 28: 681-96

51. Turk DC, Okifuji A: Psychological factors in chronic pain: evolution and revolution. J Consult Clin Psychol 2002; 70: 678-90

52. Harlow BL, Stewart EG: A population-based assessment of chronic unexplained vulvar pain: have we underestimated the prevalence of vulvodynia? J Am Med Womens Assoc 2003; 58: 82-8

53. Seckin-Alac E, Akhant SE, Bastu E, Tuzlalik S, Yavuz E: Elevated tissue levels of tumor necrosis factor-alpha in vulvar vestibulitis syndrome. Clin Exp Obstet Gynecol 2014; 41: 691-3

54. Vanderah TW: Pathophysiology of pain. Med Clin North Am 2007; 91: 1-12

55. Sahai J, Louie SG: Overview of the immune and hematopoietic systems. Am J Hosp Pharm 1993; 50: S4-9

56. Portenoy RK: Opioid therapy for chronic nonmalignant pain: a review of the critical issues. J Pain Symptom Manage 1996; 11: 203-17

57. Bell TJ, Panchal SJ, Miaskowski C, Bolge SC, Milanova T, Williamson R: The prevalence, severity, and impact of opioid-induced bowel dysfunction: results of a US and European Patient Survey (PROBE 1). Pain Med 2009; 10: 35-42

58. Benyamin R, Trescot AM, Datta S, Buenaventura R, Adlaka R, Sehgal N, Glaser SE, Vallejo R: Opioid complications and side effects. Pain Physician 2008; 11: S105-20

59. Kamen LB, Feeko KJ: Opioid Syndrome: Failed Opioid Therapy for Chronic Noncancer Pain. Phys Med Rehabil Clin N Am 2014; 25: 375-395

60. Lee M, Silverman SM, Hansen H, Patel VB, Manchikanti L: A comprehensive review of opioid-induced hyperalgesia. Pain Physician 2011; 14: 145-61

61. Wolkerstorfer A, Handler N, Buschmann H: New approaches to treating pain. Bioorg Med Chem Lett 2015

62. Chang KL, Fillingim R, Hurley RW, Schmidt S: Chronic pain management: nonpharmacological therapies for chronic pain. FP Essent 2015; 432: 21-6

63. Ehde DM, Dillworth TM, Turner JA: Cognitive-behavioral therapy for individuals with chronic pain: efficacy, innovations, and directions for research. Am Psychol 2014; 69: 153-66

64. Poobalan AS, Bruce J, King PM, Chambers WA, Krukowski ZH, Smith WC: Chronic pain and quality of life following open inguinal hernia repair. Br J Surg 2001; 88: 1122-6

65. Katz J, Jackson M, Kavanagh BP, Sandler AN: Acute pain after thoracic surgery predicts long-term post-thoracotomy pain. Clin J Pain 1996; 12: 50-5

66. Silen W: Chronic pain and quality of life following open inguinal hernia repair. Br J Surg 2002; 89: 123

67. Kehlet H, Jensen TS, Woolf CJ: Persistent postsurgical pain: risk factors and prevention. Lancet 2006; 367: 1618-25

68. de Andrade DC, Jean S, Clavelou P, Dallel R, Bouhassira D: Chronic pain associated with the Chikungunya Fever: long lasting burden of an acute illness. BMC Infect Dis 2010; 10: 31

69. Griffis CA: Neuroimmune activation and chronic pain. AANA J 2011; 79: 31-7

70. Bornstein J, Livnat G, Stolar Z, Abramovici H: Pure versus complicated vulvar vestibulitis: a randomized trial of fluconazole treatment. Gynecol Obstet Invest 2000; 50: 194-7

71. Ameratunga S, Tin ST, Connor J, Norton R: Chronic neck pain following car crashes: a population-based study from Auckland, New Zealand. Intern Med J 2010; 40: 704-9

72. Laborde JM: Biomechanics of minor automobile accidents: treatment implications for associated chronic spine symptoms. J South Orthop Assoc 2000; 9: 187-92

73. Fabricant L, Ham B, Mullins R, Mayberry J: Prolonged pain and disability are common after rib fractures. Am J Surg 2013; 205: 511-5; discusssion 515-6

74. Hosey RG, Fernandez MM, Johnson DL: Evaluation and management of stress fractures of the pelvis and sacrum. Orthopedics 2008; 31: 383-5

75. Rapkin AJ, Kames LD, Darke LL, Stampler FM, Naliboff BD: History of physical and sexual abuse in women with chronic pelvic pain. Obstet Gynecol 1990; 76: 92-6

76. Wong J, Mellor D: Intimate Partner Violence and Women's Health and Wellbeing: Impacts, risk factors and responses. Contemp Nurse 2013

77. Howard FM: Abuse history and chronic pain in women: I. Prevalences of sexual abuse and physical abuse. Obstet Gynecol 1995; 85: 158-9

78. Levy MH, Chwistek M, Mehta RS: Management of chronic pain in cancer survivors. Cancer J 2008; 14: 401-9

79. Moryl N, Coyle N, Essandoh S, Glare P: Chronic pain management in cancer survivors. J Natl Compr Canc Netw 2010; 8: 1104-10

80. van den Beuken-van Everdingen M: Chronic pain in cancer survivors: a growing issue. J Pain Palliat Care Pharmacother 2012; 26: 385-7

81. Mogil JS: Pain genetics: past, present and future. Trends Genet 2012; 28: 258-66

82. Mogil JS: The genetic mediation of individual differences in sensitivity to pain and its inhibition. Proc Natl Acad Sci U S A 1999; 96: 7744-51

83. Diatchenko L, Nackley AG, Tchivileva IE, Shabalina SA, Maixner W: Genetic architecture of human pain perception. Trends Genet 2007; 23: 605-13

84. Javierre BM, Fernandez AF, Richter J, Al-Shahrour F, Martin-Subero JI, Rodriguez-Ubreva J, Berdasco M, Fraga MF, O'Hanlon TP, Rider LG, Jacinto FV, Lopez-Longo FJ, Dopazo J, Forn M, Peinado MA, Carreno L, Sawalha AH, Harley JB, Siebert R, Esteller M, Miller FW, Ballestar E: Changes in the pattern of DNA methylation associate with twin discordance in systemic lupus erythematosus. Genome Res 2010; 20: 170-9

85. Michalowicz BS, Pihlstrom BL, Hodges JS, Bouchard TJ, Jr.: No heritability of temporomandibular joint signs and symptoms. J Dent Res 2000; 79: 1573-8

86. Buchheit T, Van de Ven T, Shaw A: Epigenetics and the transition from acute to chronic pain. Pain Med 2012; 13: 1474-90

87. Denk F, McMahon SB, Tracey I: Pain vulnerability: a neurobiological perspective. Nat Neurosci 2014; 17: 192-200

88. Doehring A, Geisslinger G, Lotsch J: Epigenetics in pain and analgesia: an imminent research field. Eur J Pain 2011; 15: 11-6

89. Lacroix-Fralish ML, Tawfik VL, Tanga FY, Spratt KF, DeLeo JA: Differential spinal cord gene expression in rodent models of radicular and neuropathic pain. Anesthesiology 2006; 104: 1283-92

90. Slade GD, Diatchenko L, Ohrbach R, Maixner W: Orthodontic Treatment, Genetic Factors and Risk of Temporomandibular Disorder. Semin Orthod 2008; 14: 146-156

91. Diatchenko L, Nackley AG, Slade GD, Fillingim RB, Maixner W: Idiopathic pain disorders--pathways of vulnerability. Pain 2006; 123: 226-30

92. Bartel DP: MicroRNAs: genomics, biogenesis, mechanism, and function. Cell 2004; 116: 281-97

93. Niederberger E, Kynast K, Lotsch J, Geisslinger G: MicroRNAs as new players in the pain game. Pain 2011; 152: 1455-8

94. Gandhi R, Healy B, Gholipour T, Egorova S, Musallam A, Hussain MS, Nejad P, Patel B, Hei H, Khoury S, Quintana F, Kivisakk P, Chitnis T, Weiner HL: Circulating microRNAs as biomarkers for disease staging in multiple sclerosis. Ann Neurol 2013; 73: 729-40

95. Kloos W, Vogel B, Blessing E: MiRNAs in peripheral artery disease - something gripping this way comes. Vasa 2014; 43: 163-70

96. Dwivedi Y: Emerging role of microRNAs in major depressive disorder: diagnosis and therapeutic implications. Dialogues Clin Neurosci 2014; 16: 43-61

97. Sayed AS, Xia K, Salma U, Yang T, Peng J: Diagnosis, Prognosis and Therapeutic Role of Circulating miRNAs in Cardiovascular Diseases. Heart Lung Circ 2014

98. Hornby RJ, Starkey Lewis P, Dear J, Goldring C, Park K: MicroRNAs as potential circulating biomarkers of drug-induced liver injury: key current and future issues for translation to humans. Expert Rev Clin Pharmacol 2014; 7: 349-62

99. Garzon R, Calin GA, Croce CM: MicroRNAs in Cancer. Annu Rev Med 2009; 60: 167-79

100. Bader AG, Brown D, Stoudemire J, Lammers P: Developing therapeutic microRNAs for cancer. Gene Ther 2011; 18: 1121-6

101. Creemers EE, Tijsen AJ, Pinto YM: Circulating microRNAs: novel biomarkers and extracellular communicators in cardiovascular disease? Circ Res 2012; 110: 483-95

102. Generaal E, Vogelzangs N, Macfarlane GJ, Geenen R, Smit JH, Dekker J, Penninx BW: Basal inflammation and innate immune response in chronic multisite musculoskeletal pain. Pain 2014

103. Marchand F, Perretti M, McMahon SB: Role of the immune system in chronic pain. Nat Rev Neurosci 2005; 6: 521-32

104. Dai R, Ahmed SA: MicroRNA, a new paradigm for understanding immunoregulation, inflammation, and autoimmune diseases. Transl Res 2011; 157: 163-79

105. Chen T, Li Z, Tu J, Zhu W, Ge J, Zheng X, Yang L, Pan X, Yan H, Zhu J: MicroRNA-29a regulates pro-inflammatory cytokine secretion and scavenger receptor expression by targeting LPL in oxLDL-stimulated dendritic cells. FEBS Lett 2011; 585: 657-63

106. Chen T, Li Z, Jing T, Zhu W, Ge J, Zheng X, Pan X, Yan H, Zhu J: MicroRNA-146a regulates the maturation process and pro-inflammatory cytokine secretion by targeting CD40L in oxLDL-stimulated dendritic cells. FEBS Lett 2011; 585: 567-73

107. Sun Y, Li Q, Gui H, Xu DP, Yang YL, Su DF, Liu X: MicroRNA-124 mediates the cholinergic anti-inflammatory action through inhibiting the production of pro-inflammatory cytokines. Cell Res 2013; 23: 1270-83

108. Xu Y, Jin H, Yang X, Wang L, Su L, Liu K, Gu Q, Xu X: MicroRNA-93 inhibits inflammatory cytokine production in LPS-stimulated murine macrophages by targeting IRAK4. FEBS Lett 2014; 588: 1692-8

109. Sun X, Icli B, Wara AK, Belkin N, He S, Kobzik L, Hunninghake GM, Vera MP, Registry M, Blackwell TS, Baron RM, Feinberg MW: MicroRNA-181b regulates NF-kappaB-mediated vascular inflammation. J Clin Invest 2012; 122: 1973-90

110. Ma X, Becker Buscaglia LE, Barker JR, Li Y: MicroRNAs in NF-kappaB signaling. J Mol Cell Biol 2011; 3: 159-66

111. Bai G, Ambalavanar R, Wei D, Dessem D: Downregulation of selective microRNAs in trigeminal ganglion neurons following inflammatory muscle pain. Mol Pain 2007; 3: 15

112. Kusuda R, Cadetti F, Ravanelli MI, Sousa TA, Zanon S, De Lucca FL, Lucas G: Differential expression of microRNAs in mouse pain models. Mol Pain 2011; 7: 17

113. Poh KW, Yeo JF, Ong WY: MicroRNA changes in the mouse prefrontal cortex after inflammatory pain. Eur J Pain 2011; 15: 801 e1-12

114. Aldrich BT, Frakes EP, Kasuya J, Hammond DL, Kitamoto T: Changes in expression of sensory organ-specific microRNAs in rat dorsal root ganglia in association with mechanical hypersensitivity induced by spinal nerve ligation. Neuroscience 2009; 164: 711-23

115. von Schack D, Agostino MJ, Murray BS, Li Y, Reddy PS, Chen J, Choe SE, Strassle BW, Li C, Bates B, Zhang L, Hu H, Kotnis S, Bingham B, Liu W, Whiteside GT, Samad TA, Kennedy JD, Ajit SK: Dynamic changes in the microRNA expression profile reveal multiple regulatory mechanisms in the spinal nerve ligation model of neuropathic pain. PLoS One 2011; 6: e17670

116. Zhao J, Lee MC, Momin A, Cendan CM, Shepherd ST, Baker MD, Asante C, Bee L, Bethry A, Perkins JR, Nassar MA, Abrahamsen B, Dickenson A, Cobb BS, Merkenschlager M, Wood JN: Small RNAs control sodium channel expression, nociceptor excitability, and pain thresholds. J Neurosci 2010; 30: 10860-71

117. Favereaux A, Thoumine O, Bouali-Benazzouz R, Roques V, Papon MA, Salam SA, Drutel G, Leger C, Calas A, Nagy F, Landry M: Bidirectional integrative regulation of Cav1.2 calcium channel by microRNA miR-103: role in pain. EMBO J 2011; 30: 3830-41

118. Willemen HL, Huo XJ, Mao-Ying QL, Zijlstra J, Heijnen CJ, Kavelaars A: MicroRNA-124 as a novel treatment for persistent hyperalgesia. J Neuroinflammation 2012; 9: 143

119. Kress M, Huttenhofer A, Landry M, Kuner R, Favereaux A, Greenberg D, Bednarik J, Heppenstall P, Kronenberg F, Malcangio M, Rittner H, Uceyler N, Trajanoski Z, Mouritzen P, Birklein F, Sommer C, Soreq H: microRNAs in nociceptive circuits as predictors of future clinical applications. Front Mol Neurosci 2013; 6: 33

120. Li X, Gibson G, Kim JS, Kroin J, Xu S, van Wijnen AJ, Im HJ: MicroRNA-146a is linked to pain-related pathophysiology of osteoarthritis. Gene 2011; 480: 34-41

121. Orlova IA, Alexander GM, Qureshi RA, Sacan A, Graziano A, Barrett JE, Schwartzman RJ, Ajit SK: MicroRNA modulation in complex regional pain syndrome. J Transl Med 2011; 9: 195

122. Cerda-Olmedo G, Mena-Duran AV, Monsalve V, Oltra E: Identification of a microRNA signature for the diagnosis of fibromyalgia. PLoS One 2015; 10: e0121903

123. Gheinani AH, Burkhard FC, Monastyrskaya K: Deciphering microRNA code in pain and inflammation: lessons from bladder pain syndrome. Cell Mol Life Sci 2013; 70: 3773-89

124. Andersen HH, Duroux M, Gazerani P: Serum MicroRNA Signatures in Migraineurs During Attacks and in Pain-Free Periods. Mol Neurobiol 2015

125. Mannisto PT, Kaakkola S: Catechol-O-methyltransferase (COMT): biochemistry, molecular biology, pharmacology, and clinical efficacy of the new selective COMT inhibitors. Pharmacol Rev 1999; 51: 593-628

126. Tenhunen J, Salminen M, Lundstrom K, Kiviluoto T, Savolainen R, Ulmanen I: Genomic organization of the human catechol O-methyltransferase gene and its expression from two distinct promoters. Eur J Biochem 1994; 223: 1049-59

127. Marbach JJ, Levitt M: Erythrocyte catechol-O-methyltransferase activity in facial pain patients. J Dent Res 1976; 55: 711

128. Smith SB, Reenila I, Mannisto PT, Slade GD, Maixner W, Diatchenko L, Nackley AG: Epistasis between polymorphisms in COMT, ESR1, and GCH1 influences COMT enzyme activity and pain. Pain 2014; 155: 2390-9

129. Huskisson EC: Catecholamine excretion and pain. Br J Clin Pharmacol 1974; 1: 80-2

130. Hasegawa M, Hada J, Fujiwara M, Honda K: Effects of repeated tooth pulp stimulation on concentrations of plasma catecholamines, corticosterone, and glucose in rats. Neurol Res 2014: 1743132813Y0000000313

131. Torpy DJ, Papanicolaou DA, Lotsikas AJ, Wilder RL, Chrousos GP, Pillemer SR: Responses of the sympathetic nervous system and the hypothalamic-pituitary-adrenal axis to interleukin-6: a pilot study in fibromyalgia. Arthritis Rheum 2000; 43: 872-80

132. Evaskus DS, Laskin DM: A biochemical measure of stress in patients with myofascial pain-dysfunction syndrome. J Dent Res 1972; 51: 1464-6

133. Perry F, Heller PH, Kamiya J, Levine JD: Altered autonomic function in patients with arthritis or with chronic myofascial pain. Pain 1989; 39: 77-84

134. Charrua A, Pinto R, Birder LA, Cruz F: Sympathetic nervous system and chronic bladder pain: a new tune for an old song. Transl Androl Urol 2015; 4: 534-542

135. Kvetnansky R, Sabban EL, Palkovits M: Catecholaminergic systems in stress: structural and molecular genetic approaches. Physiol Rev 2009; 89: 535-606

136. Player MS, Peterson LE: Anxiety disorders, hypertension, and cardiovascular risk: a review. Int J Psychiatry Med 2011; 41: 365-77

137. Januzzi JL, Jr., Stern TA, Pasternak RC, DeSanctis RW: The influence of anxiety and depression on outcomes of patients with coronary artery disease. Arch Intern Med 2000; 160: 1913-21

138. Potter WZ, Manji HK: Catecholamines in depression: an update. Clin Chem 1994; 40: 279-87

139. Rakvag TT, Klepstad P, Baar C, Kvam TM, Dale O, Kaasa S, Krokan HE, Skorpen F: The Val158Met polymorphism of the human catechol-O-methyltransferase (COMT) gene may influence morphine requirements in cancer pain patients. Pain 2005; 116: 73-8

140. Tchivileva IE, Lim PF, Smith SB, Slade GD, Diatchenko L, McLean SA, Maixner W: Effect of catechol-O-methyltransferase polymorphism on response to propranolol therapy in chronic musculoskeletal pain: a randomized, double-blind, placebo-controlled, crossover pilot study. Pharmacogenet Genomics 2010; 20: 239-48

141. Diatchenko L, Slade GD, Nackley AG, Bhalang K, Sigurdsson A, Belfer I, Goldman D, Xu K, Shabalina SA, Shagin D, Max MB, Makarov SS, Maixner W: Genetic basis for individual variations in pain perception and the development of a chronic pain condition. Hum Mol Genet 2005; 14: 135-43

142. Sadhasivam S, Chidambaran V, Olbrecht VA, Esslinger HR, Zhang K, Zhang X, Martin LJ: Genetics of pain perception, COMT and postoperative pain management in children. Pharmacogenomics 2014; 15: 277-84

143. George SZ, Wallace MR, Wright TW, Moser MW, Greenfield WH, 3rd, Sack BK, Herbstman DM, Fillingim RB: Evidence for a biopsychosocial influence on shoulder pain: pain catastrophizing and catechol-O-methyltransferase (COMT) diplotype predict clinical pain ratings. Pain 2008; 136: 53-61

144. McLean SA, Diatchenko L, Lee YM, Swor RA, Domeier RM, Jones JS, Jones CW, Reed C, Harris RE, Maixner W, Clauw DJ, Liberzon I: Catechol O-methyltransferase haplotype predicts immediate musculoskeletal neck pain and psychological symptoms after motor vehicle collision. J Pain 2011; 12: 101-7

145. Gursoy S, Erdal E, Herken H, Madenci E, Alasehirli B, Erdal N: Significance of catechol-O-methyltransferase gene polymorphism in fibromyalgia syndrome. Rheumatol Int 2003; 23: 104-7

146. Vargas-Alarcon G, Fragoso JM, Cruz-Robles D, Vargas A, Vargas A, Lao-Villadoniga JI, Garcia-Fructuoso F, Ramos-Kuri M, Hernandez F, Springall R, Bojalil R, Vallejo M, Martinez-Lavin M: Catechol-O-methyltransferase gene haplotypes in Mexican and Spanish patients with fibromyalgia. Arthritis Res Ther 2007; 9: R110

147. Nackley AG, Tan KS, Fecho K, Flood P, Diatchenko L, Maixner W: Catechol-O-methyltransferase inhibition increases pain sensitivity through activation of both beta2- and beta3-adrenergic receptors. Pain 2007; 128: 199-208

148. Yancey JR, Sheridan R, Koren KG: Chronic daily headache: diagnosis and management. Am Fam Physician 2014; 89: 642-8

149. Hartung JE, Ciszek BP, Nackley AG: beta2- and beta3-adrenergic receptors drive COMT-dependent pain by increasing production of nitric oxide and cytokines. Pain 2014; 155: 1346-55

150. Kline RHt, Exposto FG, O'Buckley SC, Westlund KN, Nackley AG: Catechol-O-methyltransferase inhibition alters pain and anxiety-related volitional behaviors through activation of beta-adrenergic receptors in the rat. Neuroscience 2015; 290: 561-9

151. Light KC, Bragdon EE, Grewen KM, Brownley KA, Girdler SS, Maixner W: Adrenergic dysregulation and pain with and without acute beta-blockade in women with fibromyalgia and temporomandibular disorder. J Pain 2009; 10: 542-52

152. Wachter SB, Gilbert EM: Beta-adrenergic receptors, from their discovery and characterization through their manipulation to beneficial clinical application. Cardiology 2012; 122: 104-12

153. Baerwald CG, Laufenberg M, Specht T, von Wichert P, Burmester GR, Krause A: Impaired sympathetic influence on the immune response in patients with rheumatoid arthritis due to lymphocyte subset-specific modulation of beta 2-adrenergic receptors. Br J Rheumatol 1997; 36: 1262-9

154. Kaplan R, Robinson CA, Scavulli JF, Vaughan JH: Propranolol and the treatment of rheumatoid arthritis. Arthritis Rheum 1980; 23: 253-5

155. Levine JD, Fye K, Heller P, Basbaum AI, Whiting-O'Keefe Q: Clinical response to regional intravenous guanethidine in patients with rheumatoid arthritis. J Rheumatol 1986; 13: 1040-3

156. Johnson M: Molecular mechanisms of beta(2)-adrenergic receptor function, response, and regulation. J Allergy Clin Immunol 2006; 117: 18-24; quiz 25

157. Strosberg AD: Structure and function of the beta 3-adrenergic receptor. Annu Rev Pharmacol Toxicol 1997; 37: 421-50

158. Soeder KJ, Snedden SK, Cao W, Della Rocca GJ, Daniel KW, Luttrell LM, Collins S: The beta3-adrenergic receptor activates mitogen-activated protein kinase in adipocytes through a Gi-dependent mechanism. J Biol Chem 1999; 274: 12017-22

159. Aley KO, Martin A, McMahon T, Mok J, Levine JD, Messing RO: Nociceptor sensitization by extracellular signal-regulated kinases. J Neurosci 2001; 21: 6933-9

160. Khasar SG, Lin YH, Martin A, Dadgar J, McMahon T, Wang D, Hundle B, Aley KO, Isenberg W, McCarter G, Green PG, Hodge CW, Levine JD, Messing RO: A novel nociceptor signaling pathway revealed in protein kinase C epsilon mutant mice. Neuron 1999; 24: 253-60

161. Khasar SG, McCarter G, Levine JD: Epinephrine produces a beta-adrenergic receptor-mediated mechanical hyperalgesia and in vitro sensitization of rat nociceptors. J Neurophysiol 1999; 81: 1104-12

162. Zhang C, Rui YY, Zhou YY, Ju Z, Zhang HH, Hu CY, Xiao Y, Xu GY: Adrenergic beta2-receptors mediates visceral hypersensitivity induced by heterotypic intermittent stress in rats. PLoS One 2014; 9: e94726

163. Khasar SG, Green PG, Miao FJ, Levine JD: Vagal modulation of nociception is mediated by adrenomedullary epinephrine in the rat. Eur J Neurosci 2003; 17: 909-15

164. Hucho TB, Dina OA, Kuhn J, Levine JD: Estrogen controls PKCepsilon-dependent mechanical hyperalgesia through direct action on nociceptive neurons. Eur J Neurosci 2006; 24: 527-34

165. Ochoa-Cortes F, Guerrero-Alba R, Valdez-Morales EE, Spreadbury I, Barajas-Lopez C, Castro M, Bertrand J, Cenac N, Vergnolle N, Vanner SJ: Chronic stress mediators act synergistically on colonic nociceptive mouse dorsal root ganglia neurons to increase excitability. Neurogastroenterol Motil 2014; 26: 334-45

166. Wang S, Zhu HY, Jin Y, Zhou Y, Hu S, Liu T, Jiang X, Xu GY: Adrenergic signaling mediates mechanical hyperalgesia through activation of P2X3 receptors in primary sensory neurons of rats with chronic pancreatitis. Am J Physiol Gastrointest Liver Physiol 2015; 308: G710-9

167. Dasu MR, Ramirez SR, La TD, Gorouhi F, Nguyen C, Lin BR, Mashburn C, Stewart H, Peavy TR, Nolta JA, Isseroff RR: Crosstalk between adrenergic and toll-like receptors in human mesenchymal stem cells and keratinocytes: a recipe for impaired wound healing. Stem Cells Transl Med 2014; 3: 745-59

168. Koizumi H, Tanaka H, Ohkawara A: beta-Adrenergic stimulation induces activation of protein kinase C and inositol 1,4,5-trisphosphate increase in epidermis. Exp Dermatol 1997; 6: 128-32

169. Pullar CE, Isseroff RR: The beta 2-adrenergic receptor activates pro-migratory and pro-proliferative pathways in dermal fibroblasts via divergent mechanisms. J Cell Sci 2006; 119: 592-602

170. Chi DS, Fitzgerald SM, Pitts S, Cantor K, King E, Lee SA, Huang SK, Krishnaswamy G: MAPK-dependent regulation of IL-1- and beta-adrenoreceptor-induced inflammatory cytokine production from mast cells: implications for the stress response. BMC Immunol 2004; 5: 22

171. Chiarella SE, Soberanes S, Urich D, Morales-Nebreda L, Nigdelioglu R, Green D, Young JB, Gonzalez A, Rosario C, Misharin AV, Ghio AJ, Wunderink RG, Donnelly HK, Radigan KA, Perlman H, Chandel NS, Budinger GR, Mutlu GM: beta(2)-Adrenergic agonists augment air pollution-induced IL-6 release and thrombosis. J Clin Invest 2014; 124: 2935-46

172. Kim MH, Gorouhi F, Ramirez S, Granick JL, Byrne BA, Soulika AM, Simon SI, Isseroff RR: Catecholamine stress alters neutrophil trafficking and impairs wound healing by

beta2-adrenergic receptor-mediated upregulation of IL-6. J Invest Dermatol 2014; 134: 809-17

173. Laukova M, Vargovic P, Csaderova L, Chovanova L, Vlcek M, Imrich R, Krizanova O, Kvetnansky R: Acute stress differently modulates beta1, beta2 and beta3 adrenoceptors in T cells, but not in B cells, from the rat spleen. Neuroimmunomodulation 2012; 19: 69-78

174. Collins S, Surwit RS: The beta-adrenergic receptors and the control of adipose tissue metabolism and thermogenesis. Recent Prog Horm Res 2001; 56: 309-28

175. Rainbow TC, Parsons B, Wolfe BB: Quantitative autoradiography of beta 1- and beta 2-adrenergic receptors in rat brain. Proc Natl Acad Sci U S A 1984; 81: 1585-9

176. Nicholson R, Dixon AK, Spanswick D, Lee K: Noradrenergic receptor mRNA expression in adult rat superficial dorsal horn and dorsal root ganglion neurons. Neurosci Lett 2005; 380: 316-21

177. Stone EA, Ariano MA: Are glial cells targets of the central noradrenergic system? A review of the evidence. Brain Res Brain Res Rev 1989; 14: 297-309

178. Kanno T, Yaguchi T, Nishizaki T: Noradrenaline stimulates ATP release from DRG neurons by targeting beta(3) adrenoceptors as a factor of neuropathic pain. J Cell Physiol 2010; 224: 345-51

179. Claustre Y, Leonetti M, Santucci V, Bougault I, Desvignes C, Rouquier L, Aubin N, Keane P, Busch S, Chen Y, Palejwala V, Tocci M, Yamdagni P, Didier M, Avenet P, Le Fur G, Oury-Donat F, Scatton B, Steinberg R: Effects of the beta3-adrenoceptor (Adrb3) agonist SR58611A (amibegron) on serotonergic and noradrenergic transmission in the rodent: relevance to its antidepressant/anxiolytic-like profile. Neuroscience 2008; 156: 353-64

180. Wang WC, Juan AH, Panebra A, Liggett SB: MicroRNA let-7 establishes expression of beta2-adrenergic receptors and dynamically down-regulates agonist-promoted down-regulation. Proc Natl Acad Sci U S A 2011; 108: 6246-51

181. Sayed D, Rane S, Lypowy J, He M, Chen IY, Vashistha H, Yan L, Malhotra A, Vatner D, Abdellatif M: MicroRNA-21 targets Sprouty2 and promotes cellular outgrowths. Mol Biol Cell 2008; 19: 3272-82

182. Rane S, He M, Sayed D, Yan L, Vatner D, Abdellatif M: An antagonism between the AKT and beta-adrenergic signaling pathways mediated through their reciprocal effects on miR-199a-5p. Cell Signal 2010; 22: 1054-62

183. Lu Y, Zhang Y, Shan H, Pan Z, Li X, Li B, Xu C, Zhang B, Zhang F, Dong D, Song W, Qiao G, Yang B: MicroRNA-1 downregulation by propranolol in a rat model of myocardial infarction: a new mechanism for ischaemic cardioprotection. Cardiovasc Res 2009; 84: 434-41

184. Funahashi H, Izawa H, Hirashiki A, Cheng XW, Inden Y, Nomura M, Murohara T: Altered microRNA expression associated with reduced catecholamine sensitivity in patients with chronic heart failure. J Cardiol 2011; 57: 338-44

185. Clauw DJ: Fibromyalgia: a clinical review. JAMA 2014; 311: 1547-55

186. Dzau VJ, Pizzo PA: Relieving pain in America: insights from an Institute of Medicine committee. JAMA 2014; 312: 1507-8

187. Rey E, Talley NJ: Irritable bowel syndrome: novel views on the epidemiology and potential risk factors. Dig Liver Dis 2009; 41: 772-80

188. Slade GD, Bair E, Greenspan JD, Dubner R, Fillingim RB, Diatchenko L, Maixner W, Knott C, Ohrbach R: Signs and symptoms of first-onset TMD and sociodemographic predictors of its development: the OPPERA prospective cohort study. J Pain 2013; 14: T20-32 e1-3

189. Tsang A, Von Korff M, Lee S, Alonso J, Karam E, Angermeyer MC, Borges GL, Bromet EJ, Demytteneare K, de Girolamo G, de Graaf R, Gureje O, Lepine JP, Haro JM, Levinson D, Oakley Browne MA, Posada-Villa J, Seedat S, Watanabe M: Common chronic pain conditions in developed and developing countries: gender and age differences and comorbidity with depression-anxiety disorders. J Pain 2008; 9: 883-91

190. Wesselmann U, Bonham A, Foster D: Vulvodynia: Current state of the biological science. Pain 2014; 155: 1696-701

191. Institute of Medicine (U.S.). Committee on Advancing Pain Research Care and Education.: Relieving pain in America : a blueprint for transforming prevention, care, education, and research. Washington, D.C., National Academies Press, 2011

192. Smith SB, Maixner DW, Greenspan JD, Dubner R, Fillingim RB, Ohrbach R, Knott C, Slade GD, Bair E, Gibson DG, Zaykin DV, Weir BS, Maixner W, Diatchenko L: Potential genetic risk factors for chronic TMD: genetic associations from the OPPERA case control study. J Pain 2011; 12: T92-101

193. Capellino S, Cosentino M, Wolff C, Schmidt M, Grifka J, Straub RH: Catecholamine-producing cells in the synovial tissue during arthritis: modulation of sympathetic neurotransmitters as new therapeutic target. Ann Rheum Dis 2010; 69: 1853-60

194. Flierl MA, Rittirsch D, Huber-Lang M, Sarma JV, Ward PA: Catecholamines-crafty weapons in the inflammatory arsenal of immune/inflammatory cells or opening pandora's box? Mol Med 2008; 14: 195-204

195. Harden RN, Rudin NJ, Bruehl S, Kee W, Parikh DK, Kooch J, Duc T, Gracely RH: Increased systemic catecholamines in complex regional pain syndrome and relationship to psychological factors: a pilot study. Anesth Analg 2004; 99: 1478-85; table of contents

196. Lotta T, Vidgren J, Tilgmann C, Ulmanen I, Melen K, Julkunen I, Taskinen J: Kinetics of human soluble and membrane-bound catechol O-methyltransferase: a revised mechanism and description of the thermolabile variant of the enzyme. Biochemistry 1995; 34: 4202-10

197. Nackley AG, Shabalina SA, Tchivileva IE, Satterfield K, Korchynskyi O, Makarov SS, Maixner W, Diatchenko L: Human catechol-O-methyltransferase haplotypes modulate protein expression by altering mRNA secondary structure. Science 2006; 314: 1930-3

198. Barbosa FR, Matsuda JB, Mazucato M, de Castro Franca S, Zingaretti SM, da Silva LM, Martinez-Rossi NM, Junior MF, Marins M, Fachin AL: Influence of catechol-O-methyltransferase (COMT) gene polymorphisms in pain sensibility of Brazilian fibromialgia patients. Rheumatol Int 2012; 32: 427-30

199. Cohen H, Neumann L, Glazer Y, Ebstein RP, Buskila D: The relationship between a common catechol-O-methyltransferase (COMT) polymorphism val(158) met and fibromyalgia. Clin Exp Rheumatol 2009; 27: S51-6

200. Matsuda JB, Barbosa FR, Morel LJ, Franca Sde C, Zingaretti SM, da Silva LM, Pereira AM, Marins M, Fachin AL: Serotonin receptor (5-HT 2A) and catechol-O-methyltransferase (COMT) gene polymorphisms: triggers of fibromyalgia? Rev Bras Reumatol 2010; 50: 141-9

201. Zubieta JK, Heitzeg MM, Smith YR, Bueller JA, Xu K, Xu Y, Koeppe RA, Stohler CS, Goldman D: COMT val158met genotype affects mu-opioid neurotransmitter responses to a pain stressor. Science 2003; 299: 1240-3

202. Mobascher A, Brinkmeyer J, Thiele H, Toliat MR, Steffens M, Warbrick T, Musso F, Wittsack HJ, Saleh A, Schnitzler A, Winterer G: The val158met polymorphism of human catechol-O-methyltransferase (COMT) affects anterior cingulate cortex activation in response to painful laser stimulation. Mol Pain 2010; 6: 32

203. Barbosa F, Matsuda J, Mazucato M, de Castro França S, Zingaretti S, da Silva L, Martinez-Rossi N, Júnior M, Marins M, Fachin A: Influence of catechol-O-methyltransferase (COMT) gene polymorphisms in pain sensibility of Brazilian fibromialgia patients. Rheumatology International 2012; 32: 427-430

204. Desmeules J, Chabert J, Rebsamen M, Rapiti E, Piguet V, Besson M, Dayer P, Cedraschi C: Central pain sensitization, COMT Val158Met polymorphism, and emotional factors in fibromyalgia. J Pain 2014; 15: 129-35

205. Ren K, Dubner R: Inflammatory Models of Pain and Hyperalgesia. ILAR J 1999; 40: 111-118

206. Kim KJ, Yoon YW, Chung JM: Comparison of three rodent neuropathic pain models. Exp Brain Res 1997; 113: 200-6

207. Martin LJ, Hathaway G, Isbester K, Mirali S, Acland EL, Niederstrasser N, Slepian PM, Trost Z, Bartz JA, Sapolsky RM, Sternberg WF, Levitin DJ, Mogil JS: Reducing social stress elicits emotional contagion of pain in mouse and human strangers. Curr Biol 2015; 25: 326-32

208. Lariviere WR, Mogil JS: The genetics of pain and analgesia in laboratory animals. Methods Mol Biol 2010; 617: 261-78

209. Haddad F, Adams GR: Inhibition of MAP/ERK kinase prevents IGF-I-induced hypertrophy in rat muscles. J Appl Physiol (1985) 2004; 96: 203-10

210. Yaksh TL, Rudy TA: Chronic catheterization of the spinal subarachnoid space. Physiol Behav 1976; 17: 1031-6

211. DeVos SL, Miller TM: Direct intraventricular delivery of drugs to the rodent central nervous system. J Vis Exp 2013: e50326

212. Chaplan SR, Bach FW, Pogrel JW, Chung JM, Yaksh TL: Quantitative assessment of tactile allodynia in the rat paw. J Neurosci Methods 1994; 53: 55-63

213. Hargreaves K, Dubner R, Brown F, Flores C, Joris J: A new and sensitive method for measuring thermal nociception in cutaneous hyperalgesia. Pain 1988; 32: 77-88

214. Dumka VK, Tandan SK, Raviprakash V, Tripathi HC: Central noradrenergic and cholinergic modulation of formaldehyde-induced pedal inflammation and nociception in rats. Indian J Physiol Pharmacol 1996; 40: 41-6

215. Fukui M, Nakagawa T, Minami M, Satoh M: Involvement of beta2-adrenergic and mu-opioid receptors in antinociception produced by intracerebroventricular administration of alpha,beta-methylene-ATP. Jpn J Pharmacol 2001; 86: 423-8

216. Kanzler SA, Januario AC, Paschoalini MA: Involvement of beta3-adrenergic receptors in the control of food intake in rats. Braz J Med Biol Res 2011; 44: 1141-7

217. Kubota E, Kubota T, Matsumoto J, Shibata T, Murakami KI: Synovial fluid cytokines and proteinases as markers of temporomandibular joint disease. J Oral Maxillofac Surg 1998; 56: 192-198

218. Uzar E, Evliyaoglu O, Yucel Y, Ugur Cevik M, Acar A, Guzel I, Islamoglu Y, Colpan L, Tasdemir N: Serum cytokine and pro-brain natriuretic peptide (BNP) levels in pateints with migraine. Eur Rev Med Pharmacol Sci 2011; 10: 1111-1116

219. Sarchielli P, Alberti A, Baldi A, Coppola F, Rossi C, Pierguidi L, Floridi A, Calabresi P: Proinflammatory cytokines, adhesion molecules, and lymphocyte integrin expression in the internal jugular blood of migraine patients without aura assessed ictally. Headache 2006; 46: 200-7

220. Omote K, Hazama K, Kawamata T, Kawamata M, Nakayaka Y, Toriyabe M, Namiki A: Peripheral nitric oxide in carrageenan-induced inflammation. Brain Res 2001: 171-175

221. Holguin A, O'Connor KA, Biedenkapp J, Campisi J, Wieseler-Frank J, Milligan ED, Hansen MK, Spataro L, Maksimova E, Bravmann C, Martin D, Fleshner M, Maier SF, Watkins LR: HIV-1 gp120 stimulates proinflammatory cytokine-mediated pain facilitation via activation of nitric oxide synthase-I (nNOS). Pain 2004; 110: 517-30

222. Kuboyama K, Tsuda M, Tsutsui M, Toyohara Y, Tozaki-Saitoh H, Shimokawa H, Yanagihara N, Inoue K: Reduced spinal microglial activation and neuropathic pain after nerve injury in mice lacking all three nitric oxide synthases. Mol Pain 2011; 7: 50

223. Boehning D, Snyder SH: Novel neural modulators. Annu Rev Neurosci 2003; 26: 105-31

224. Aley KO, McCarter G, Levine JD: Nitric oxide signaling in pain and nociceptor sensitization in the rat. J Neurosci 1998; 18: 7008-7014

225. Czeschik JC, Hagenacker T, Schafers M, Busselberg D: TNF-alpha differentially modulates ion channels of nociceptive neurons. Neurosci Lett 2008; 434: 293-8

226. Binshtok AM, Wang H, Zimmermann K, Amaya F, Vardeh D, Shi L, Brenner GJ, Ji RR, Bean BP, Woolf CJ, Samad TA: Nociceptors are interleukin-1β sensors. J Neurosci 2008; 28: 14062-73

227. Obreja O, Biasio W, Andratsch M, Lips KS, Rathee PK, Ludwig A, Rose-John S, Kress M: Fast modulation of heat-activated ionic current by proinflammatory interleukin 6 in rat sensory neurons. Brain 2005; 128: 1634-41

228. Obreja O, Rathee PK, Lips KS, Distler C, Kress M: IL-1Ɓ potentiates heat-activated currents in rat sensory neurons: involvement of IL-1RI, tyrosine kinase, and protein kinase C. FASEB 2002; 16: 1497-1503

229. Jung H, Toth PT, White FA, Miller RJ: Monocyte chemoattractant protein-1 functions as a neuromodulator in dorsal root ganglia neurons. J Neurochem 2008; 104: 254-263

230. Sun JH, Yang B, Donnelly DF, Ma C, LaMotte RH: MCP-1 enhances excitability of nociceptive neurons in chronically compressed dorsal root ganglia. J Neurophysiol 2006; 96: 2189-99

231. Vissers KC, De Jongh RF, Crul BJ, Vinken P, Meert TF: Adrenalectomy affects pain behavior of rats after formalin injection. Life Sci 2004; 74: 1243-51

232. Wang S, Lim G, Zeng Q, Sung B, Ai Y, Guo G, Yang L, Mao J: Expression of central glucocorticoid receptors after peripheral nerve injury contributes to neuropathic pain behaviors in rats. J Neurosci 2004; 24: 8595-605

233. Marinelli M, Piazza PV, Deroche V, Maccari S, Le Moal M, Simon H: Corticosterone circadian secretion differentially facilitates dopamine-mediated psychomotor effect of cocaine and morphine. J Neurosci 1994; 14: 2724-31

234. Taylor BK, Akana SF, Peterson MA, Dallman MF, Basbaum AI: Pituitary-adrenocortical responses to persistent noxious stimuli in the awake rat: endogenous corticosterone does not reduce nociception in the formalin test. Endocrinology 1998; 139: 2407-13

235. Sorge RE, Martin LJ, Isbester KA, Sotocinal SG, Rosen S, Tuttle AH, Wieskopf JS, Acland EL, Dokova A, Kadoura B, Leger P, Mapplebeck JC, McPhail M, Delaney A, Wigerblad G, Schumann AP, Quinn T, Frasnelli J, Svensson CI, Sternberg WF, Mogil JS: Olfactory exposure to males, including men, causes stress and related analgesia in rodents. Nat Methods 2014; 11: 629-32

236. Wang S, Lim G, Zeng Q, Sung B, Yang L, Mao J: Central glucocorticoid receptors modulate the expression and function of spinal NMDA receptors after peripheral nerve injury. J Neurosci 2005; 25: 488-95

237. Wang S, Lim G, Yang L, Sung B, Mao J: Downregulation of spinal glutamate transporter EAAC1 following nerve injury is regulated by central glucocorticoid receptors in rats. Pain 2006; 120: 78-85

238. Gogos JA, Morgan M, Luine V, Santha M, Ogawa S, Pfaff D, Karayiorgou M: Catechol-O-methyltransferase-deficient mice exhibit sexually dimorphic changes in catecholamine levels and behavior. Proc Natl Acad Sci U S A 1998; 95: 9991-6

239. Papaleo F, Crawley JN, Song J, Lipska BK, Pickel J, Weinberger DR, Chen J: Genetic dissection of the role of catechol-O-methyltransferase in cognition and stress reactivity in mice. J Neurosci 2008; 28: 8709-23

240. Harrison PJ, Tunbridge EM: Catechol-O-methyltransferase (COMT): a gene contributing to sex differences in brain function, and to sexual dimorphism in the predisposition to psychiatric disorders. Neuropsychopharmacology 2008; 33: 3037-45

241. White TP, Loth E, Rubia K, Krabbendam L, Whelan R, Banaschewski T, Barker GJ, Bokde AL, Buchel C, Conrod P, Fauth-Buhler M, Flor H, Frouin V, Gallinat J, Garavan H, Gowland P, Heinz A, Ittermann B, Lawrence C, Mann K, Paillere ML, Nees F, Paus T, Pausova Z, Rietschel M, Robbins T, Smolka MN, Shergill SS, Schumann G, Consortium I: Sex differences in COMT polymorphism effects on prefrontal inhibitory control in adolescence. Neuropsychopharmacology 2014; 39: 2560-9

242. Belfer I, Segall SK, Lariviere WR, Smith SB, Dai F, Slade GD, Rashid NU, Mogil JS, Campbell CM, Edwards RR, Liu Q, Bair E, Maixner W, Diatchenko L: Pain modality- and sex-specific effects of COMT genetic functional variants. Pain 2013; 154: 1368-76

243. Boudikova B, Szumlanski C, Maidak B, Weinshilboum R: Human liver catechol-O-methyltransferase pharmacogenetics. Clin Pharmacol Ther 1990; 48: 381-9

244. Chen LX, Fang Q, Chen Q, Guo J, Wang ZZ, Chen Y, Wang R: Study in vitro and in vivo of nociceptin/orphanin FQ(1-13)NH2 analogues substituting N-Me-Gly for Gly2 or Gly3. Peptides 2004; 25: 1349-54

245. Diatchenko L, Anderson AD, Slade GD, Fillingim RB, Shabalina SA, Higgins TJ, Sama S, Belfer I, Goldman D, Max MB, Weir BS, Maixner W: Three major haplotypes of the beta2 adrenergic receptor define psychological profile, blood pressure, and the risk for development of a common musculoskeletal pain disorder. Am J Med Genet B Neuropsychiatr Genet 2006; 141B: 449-62

246. Kanno T, Yaguchi T, Nishizaki T: Noradrenaline stimulates ATP release from DRG neurons by targeting beta(3) adrenoceptors as a factor of neuropathic pain. J Cell Physiol 2010; 224: 345-51

247. Favaro-Moreira NC, Parada CA, Tambeli CH: Blockade of beta(1)-, beta(2)- and beta(3)-adrenoceptors in the temporomandibular joint induces antinociception especially in female rats. Eur J Pain 2012; 16: 1302-10

248. Liggett SB, Freedman NJ, Schwinn DA, Lefkowitz RJ: Structural basis for receptor subtype-specific regulation revealed by a chimeric beta 3/beta 2-adrenergic receptor. Proc Natl Acad Sci U S A 1993; 90: 3665-9

249. Cao W, Luttrell LM, Medvedev AV, Pierce KL, Daniel KW, Dixon TM, Lefkowitz RJ, Collins S: Direct binding of activated c-Src to the beta 3-adrenergic receptor is required for MAP kinase activation. J Biol Chem 2000; 275: 38131-4

250. Slota C, Shi A, Chen G, Bevans M, Weng NP: Norepinephrine preferentially modulates memory CD8 T cell function inducing inflammatory cytokine production and reducing proliferation in response to activation. Brain Behav Immun 2015; 46: 168-79

251. Costigan M, Moss A, Latremoliere A, Johnston C, Verma-Gandhu M, Herbert TA, Barrett L, Brenner GJ, Vardeh D, Woolf CJ, Fitzgerald M: T-cell infiltration and signaling in the adult dorsal spinal cord is a major contributor to neuropathic pain-like hypersensitivity. J Neurosci 2009; 29: 14415-22

252. Vicuna L, Strochlic DE, Latremoliere A, Bali KK, Simonetti M, Husainie D, Prokosch S, Riva P, Griffin RS, Njoo C, Gehrig S, Mall MA, Arnold B, Devor M, Woolf CJ, Liberles SD, Costigan M, Kuner R: The serine protease inhibitor SerpinA3N attenuates neuropathic pain by inhibiting T cell-derived leukocyte elastase. Nat Med 2015; 21: 518-23

253. Russell IJ, Vipraio GA, Michalek JE, Craig FE, Kang YK, Richards AB: Lymphocyte markers and natural killer cell activity in fibromyalgia syndrome: effects of low-dose, sublingual use of human interferon-alpha. J Interferon Cytokine Res 1999; 19: 969-78

254. Feng B, La JH, Schwartz ES, Gebhart GF: Irritable bowel syndrome: methods, mechanisms, and pathophysiology. Neural and neuro-immune mechanisms of visceral hypersensitivity in irritable bowel syndrome. Am J Physiol Gastrointest Liver Physiol 2012; 302: G1085-98

255. Graziottin A, Skaper SD, Fusco M: Mast cells in chronic inflammation, pelvic pain and depression in women. Gynecol Endocrinol 2014; 30: 472-7

256. Levy D: Migraine pain, meningeal inflammation, and mast cells. Curr Pain Headache Rep 2009; 13: 237-40

257. Petra AI, Panagiotidou S, Stewart JM, Conti P, Theoharides TC: Spectrum of mast cell activation disorders. Expert Rev Clin Immunol 2014; 10: 729-39

258. Regauer S, Eberz B, Beham-Schmid C: Mast cell infiltrates in vulvodynia represent secondary and idiopathic mast cell hyperplasias. APMIS 2015; 123: 452-6

259. Zhang Z, Cherryholmes G, Mao A, Marek C, Longmate J, Kalos M, Amand RP, Shively JE: High plasma levels of MCP-1 and eotaxin provide evidence for an immunological basis of fibromyalgia. Exp Biol Med (Maywood) 2008; 233: 1171-80

260. Vieira-Potter VJ: Inflammation and macrophage modulation in adipose tissues. Cell Microbiol 2014; 16: 1484-92

261. Mohamed-Ali V, Goodrick S, Rawesh A, Katz DR, Miles JM, Yudkin JS, Klein S, Coppack SW: Subcutaneous adipose tissue releases interleukin-6, but not tumor necrosis factor-alpha, in vivo. J Clin Endocrinol Metab 1997; 82: 4196-200

262. Mohamed-Ali V, Flower L, Sethi J, Hotamisligil G, Gray R, Humphries SE, York DA, Pinkney J: beta-Adrenergic regulation of IL-6 release from adipose tissue: in vivo and in vitro studies. J Clin Endocrinol Metab 2001; 86: 5864-9

263. Fu L, Isobe K, Zeng Q, Suzukawa K, Takekoshi K, Kawakami Y: beta-adrenoceptor agonists downregulate adiponectin, but upregulate adiponectin receptor 2 and tumor necrosis factor-alpha expression in adipocytes. Eur J Pharmacol 2007; 569: 155-62

264. Kralisch S, Klein J, Lossner U, Bluher M, Paschke R, Stumvoll M, Fasshauer M: Isoproterenol stimulates monocyte chemoattractant protein-1 expression and secretion in 3T3-L1 adipocytes. Regul Pept 2006; 135: 12-6

265. Canova NK, Lincova D, Kmonickova E, Kamenikova L, Farghali H: Nitric oxide production from rat adipocytes is modulated by beta3-adrenergic receptor agonists and is involved in a cyclic AMP-dependent lipolysis in adipocytes. Nitric Oxide 2006; 14: 200-11

266. Gilman AG, Nirenberg M: Effect of catecholamines on the adenosine 3':5'-cyclic monophosphate concentrations of clonal satellite cells of neurons. Proc Natl Acad Sci U S A 1971; 68: 2165-8

267. Wood PB, Kablinger AS, Caldito GS: Open trial of pindolol in the treatment of fibromyalgia. Ann Pharmacother 2005; 39: 1812-6

268. Rodrigues WF, Madeira MF, da Silva TA, Clemente-Napimoga JT, Miguel CB, Dias-da-Silva VJ, Barbosa-Neto O, Lopes AH, Napimoga MH: Low dose of propranolol down-modulates bone resorption by inhibiting inflammation and osteoclast differentiation. Br J Pharmacol 2012; 165: 2140-51

269. Park JM, Mun JH, Song M, Kim HS, Kim BS, Kim MB, Ko HC: Propranolol, doxycycline and combination therapy for the treatment of rosacea. J Dermatol 2015; 42: 64-9

270. Hogan CJ, Hurwitz SR: Treatment of complex regional pain syndrome of the lower extremity. J Am Acad Orthop Surg 2002; 10: 281-9

271. Millan MJ: Descending control of pain. Prog Neurobiol 2002; 66: 355-474

272. Sanchez C, Hyttel J: Comparison of the effects of antidepressants and their metabolites on reuptake of biogenic amines and on receptor binding. Cell Mol Neurobiol 1999; 19: 467-89

273. Li W, Shi X, Wang L, Guo T, Wei T, Cheng K, Rice KC, Kingery WS, Clark JD: Epidermal adrenergic signaling contributes to inflammation and pain sensitization in a rat model of complex regional pain syndrome. Pain 2013; 154: 1224-36

274. Ciszek BP, O'Buckley SC, Nackley AG: Persistent catechol-O-methyltransferase-dependent pain is initiated by peripheral beta-adrenergic receptors. Anesthesiology 2016; In Press

275. Kagan L, Abraham AK, Harrold JM, Mager DE: Interspecies scaling of receptor-mediated pharmacokinetics and pharmacodynamics of type I interferons. Pharm Res 2010; 27: 920-32

276. Walsh J, Tighe O, Lai D, Harvey R, Karayiorgou M, Gogos JA, Waddington JL, O'Tuathaigh CM: Disruption of thermal nociceptive behaviour in mice mutant for the schizophrenia-associated genes NRG1, COMT and DISC1. Brain Res 2010; 1348: 114-9

277. Tammimaki A, Kaenmaki M, Kambur O, Kulesskaya N, Keisala T, Karvonen E, Garcia-Horsman JA, Rauvala H, Mannisto PT: Effect of S-COMT deficiency on behavior and extracellular brain dopamine concentrations in mice. Psychopharmacology (Berl) 2010; 211: 389-401

278. Papaleo F, Erickson L, Liu G, Chen J, Weinberger DR: Effects of sex and COMT genotype on environmentally modulated cognitive control in mice. Proc Natl Acad Sci U S A 2012; 109: 20160-5

279. Risbrough V, Ji B, Hauger R, Zhou X: Generation and characterization of humanized mice carrying COMT158 Met/Val alleles. Neuropsychopharmacology 2014; 39: 1823-32

280. Stubelius A, Wilhelmson AS, Gogos JA, Tivesten A, Islander U, Carlsten H: Sexual dimorphisms in the immune system of catechol-O-methyltransferase knockout mice. Immunobiology 2012; 217: 751-60

281. Allen KD, Griffin TM, Rodriguiz RM, Wetsel WC, Kraus VB, Huebner JL, Boyd LM, Setton LA: Decreased physical function and increased pain sensitivity in mice deficient for type IX collagen. Arthritis Rheum 2009; 60: 2684-93

282. Kehl LJ, Trempe TM, Hargreaves KM: A new animal model for assessing mechanisms and management of muscle hyperalgesia. Pain 2000; 85: 333-43

283. Deacon RM: Measuring the strength of mice. J Vis Exp 2013

284. Rogers DC, Fisher EM, Brown SD, Peters J, Hunter AJ, Martin JE: Behavioral and functional analysis of mouse phenotype: SHIRPA, a proposed protocol for comprehensive phenotype assessment. Mamm Genome 1997; 8: 711-3

285. Hunter AJ, Hatcher J, Virley D, Nelson P, Irving E, Hadingham SJ, Parsons AA: Functional assessments in mice and rats after focal stroke. Neuropharmacology 2000; 39: 806-16

286. Anderson KD, Gunawan A, Steward O: Quantitative assessment of forelimb motor function after cervical spinal cord injury in rats: relationship to the corticospinal tract. Exp Neurol 2005; 194: 161-74

287. Mogil JS, Graham AC, Ritchie J, Hughes SF, Austin JS, Schorscher-Petcu A, Langford DJ, Bennett GJ: Hypolocomotion, asymmetrically directed behaviors (licking, lifting, flinching, and shaking) and dynamic weight bearing (gait) changes are not measures of neuropathic pain in mice. Mol Pain 2010; 6: 34

288. Little CB, Zaki S: What constitutes an "animal model of osteoarthritis"--the need for consensus? Osteoarthritis Cartilage 2012; 20: 261-7

289. Langford DJ, Bailey AL, Chanda ML, Clarke SE, Drummond TE, Echols S, Glick S, Ingrao J, Klassen-Ross T, Lacroix-Fralish ML, Matsumiya L, Sorge RE, Sotocinal SG,

Tabaka JM, Wong D, van den Maagdenberg AM, Ferrari MD, Craig KD, Mogil JS: Coding of facial expressions of pain in the laboratory mouse. Nat Methods 2010; 7: 447-9

290. Ebert SN, Thompson RP: Embryonic epinephrine synthesis in the rat heart before innervation: association with pacemaking and conduction tissue development. Circ Res 2001; 88: 117-24

291. Yamasaki I, Takagi T, Oikawa D, Koutoku T, Koga Y, Tomonaga S, Tachibana T, Denbow MD, Furuse M: Changes in catecholamines and dopaminergic metabolites in pigeon brain during development from the late embryonic stage toward hatch. Zoolog Sci 2003; 20: 551-5

292. Ebert SN, Taylor DG: Catecholamines and development of cardiac pacemaking: an intrinsically intimate relationship. Cardiovasc Res 2006; 72: 364-74

293. Blair C: Stress and the Development of Self-Regulation in Context. Child Dev Perspect 2010; 4: 181-188

294. Gangadharan V, Kuner R: Pain hypersensitivity mechanisms at a glance. Dis Model Mech 2013; 6: 889-95

295. Gold MS, Gebhart GF: Nociceptor sensitization in pain pathogenesis. Nat Med 2010; 16: 1248-57

296. Julius D, Basbaum AI: Molecular mechanisms of nociception. Nature 2001; 413: 203-10

297. Bartel DP: MicroRNAs: target recognition and regulatory functions. Cell 2009; 136: 215-33

298. Soifer HS, Rossi JJ, Saetrom P: MicroRNAs in disease and potential therapeutic applications. Mol Ther 2007; 15: 2070-9

299. Zolnoun DA, Rohl J, Moore CG, Perinetti-Liebert C, Lamvu GM, Maixner W: Overlap between orofacial pain and vulvar vestibulitis syndrome. Clin J Pain 2008; 24: 187-91

300. Wesselmann U, Bonham A, Foster D: Vulvodynia: Current state of the biological science. Pain 2014

301. Williams RE, Hartmann KE, Sandler RS, Miller WC, Steege JF: Prevalence and characteristics of irritable bowel syndrome among women with chronic pelvic pain. Obstet Gynecol 2004; 104: 452-8

302. Brennan F, Carr DB, Cousins M: Pain management: a fundamental human right. Anesth Analg 2007; 105: 205-21

303. O'Connor RM, Dinan TG, Cryan JF: Little things on which happiness depends: microRNAs as novel therapeutic targets for the treatment of anxiety and depression. Mol Psychiatry 2012; 17: 359-76

304. Borgonio Cuadra VM, Gonzalez-Huerta NC, Romero-Cordoba S, Hidalgo-Miranda A, Miranda-Duarte A: Altered expression of circulating microRNA in plasma of patients with primary osteoarthritis and in silico analysis of their pathways. PLoS One 2014; 9: e97690

305. Iliopoulos D, Malizos KN, Oikonomou P, Tsezou A: Integrative microRNA and proteomic approaches identify novel osteoarthritis genes and their collaborative metabolic and inflammatory networks. PLoS One 2008; 3: e3740

306. Zolnoun D, Bair E, Essick G, Gracely R, Goyal V, Maixner W: Reliability and reproducibility of novel methodology for assessment of pressure pain sensitivity in pelvis. J Pain 2012; 13: 910-20

307. Pukall CF, Binik YM, Khalife S: A new instrument for pain assessment in vulvar vestibulitis syndrome. J Sex Marital Ther 2004; 30: 69-78

308. Thompson WG, Longstreth GF, Drossman DA, Heaton KW, Irvine EJ, Muller-Lissner SA: Functional bowel disorders and functional abdominal pain. Gut 1999; 45 Suppl 2: II43-7

309. Wolfe F, Clauw DJ, Fitzcharles MA, Goldenberg DL, Katz RS, Mease P, Russell AS, Russell IJ, Winfield JB, Yunus MB: The American College of Rheumatology preliminary diagnostic criteria for fibromyalgia and measurement of symptom severity. Arthritis Care Res (Hoboken) 2010; 62: 600-10

310. Dworkin SF: Research Diagnostic criteria for Temporomandibular Disorders: current status & future relevance. J Oral Rehabil 2010; 37: 734-43

311. Olesen J: The International Classification of Headache Disorders, 2nd edition: application to practice. Funct Neurol 2005; 20: 61-8

312. Price DD, Hu JW, Dubner R, Gracely RH: Peripheral suppression of first pain and central summation of second pain evoked by noxious heat pulses. Pain 1977; 3: 57-68

313. Gandek B, Ware JE, Aaronson NK, Apolone G, Bjorner JB, Brazier JE, Bullinger M, Kaasa S, Leplege A, Prieto L, Sullivan M: Cross-validation of item selection and scoring for the SF-12 Health Survey in nine countries: results from the IQOLA Project. International Quality of Life Assessment. J Clin Epidemiol 1998; 51: 1171-8

314. Kennedy BL, Morris RL, Pedley LL, Schwab JJ: The ability of the Symptom Checklist SCL-90 to differentiate various anxiety and depressive disorders. Psychiatr Q 2001; 72: 277-88

315. Chapman CR, Tuckett RP, Song CW: Pain and stress in a systems perspective: reciprocal neural, endocrine, and immune interactions. J Pain 2008; 9: 122-45

316. Sullivan PF, Fan C, Perou CM: Evaluating the comparability of gene expression in blood and brain. Am J Med Genet B Neuropsychiatr Genet 2006; 141: 261-8

317. Zeng L, Liu J, Wang Y, Wang L, Weng S, Tang Y, Zheng C, Cheng Q, Chen S, Yang GY: MicroRNA-210 as a novel blood biomarker in acute cerebral ischemia. Front Biosci (Elite Ed) 2011; 3: 1265-72

318. Mestdagh P, Van Vlierberghe P, De Weer A, Muth D, Westermann F, Speleman F, Vandesompele J: A novel and universal method for microRNA RT-qPCR data normalization. Genome Biol 2009; 10: R64

319. Huber W, von Heydebreck A, Sultmann H, Poustka A, Vingron M: Variance stabilization applied to microarray data calibration and to the quantification of differential expression. Bioinformatics 2002; 18 Suppl 1: S96-104

320. Vlachos IS, Kostoulas N, Vergoulis T, Georgakilas G, Reczko M, Maragkakis M, Paraskevopoulou MD, Prionidis K, Dalamagas T, Hatzigeorgiou AG: DIANA miRPath v.2.0: investigating the combinatorial effect of microRNAs in pathways. Nucleic Acids Res 2012; 40: W498-504

321. Lang R, Hammer M, Mages J: DUSP meet immunology: dual specificity MAPK phosphatases in control of the inflammatory response. J Immunol 2006; 177: 7497-504

322. Zolnoun D, Hartmann K, Lamvu G, As-Sanie S, Maixner W, Steege J: A conceptual model for the pathophysiology of vulvar vestibulitis syndrome. Obstet Gynecol Surv 2006; 61: 395-401; quiz 423

323. Slade GD, Smith SB, Zaykin DV, Tchivileva IE, Gibson DG, Yuryev A, Mazo I, Bair E, Fillingim R, Ohrbach R, Greenspan J, Maixner W, Diatchenko L: Facial pain with localized and widespread manifestations: separate pathways of vulnerability. Pain 2013; 154: 2335-43

324. Gracely RH, Grant MA, Giesecke T: Evoked pain measures in fibromyalgia. Best Pract Res Clin Rheumatol 2003; 17: 593-609

325. Pfau DB, Rolke R, Nickel R, Treede RD, Daublaender M: Somatosensory profiles in subgroups of patients with myogenic temporomandibular disorders and Fibromyalgia Syndrome. Pain 2009; 147: 72-83

326. Kaneyama K, Segami N, Nishimura M, Suzuki T, Sato J: Importance of proinflammatory cytokines in synovial fluid from 121 joints with temporomandibular disorders. Br J Oral Maxillofac Surg 2002; 40: 418-23

327. Bazzichi L, Rossi A, Massimetti G, Giannaccini G, Giuliano T, De Feo F, Ciapparelli A, Dell'Osso L, Bombardieri S: Cytokine patterns in fibromyalgia and their correlation with clinical manifestations. Clin Exp Rheumatol 2007; 25: 225-30

328. Hattar K, Fink L, Fietzner K, Himmel B, Grimminger F, Seeger W, Sibelius U: Cell density regulates neutrophil IL-8 synthesis: role of IL-1 receptor antagonist and soluble TNF receptors. J Immunol 2001; 166: 6287-93

329. Schwarz YA, Amin RS, Stark JM, Trapnell BC, Wilmott RW: Interleukin-1 receptor antagonist inhibits interleukin-8 expression in A549 respiratory epithelial cells infected in vitro with a replication-deficient recombinant adenovirus vector. Am J Respir Cell Mol Biol 1999; 21: 388-94

330. He N, Zheng H, Li P, Zhao Y, Zhang W, Song F, Chen K: miR-485-5p binding site SNP rs8752 in HPGD gene is associated with breast cancer risk. PLoS One 2014; 9: e102093

331. Zaman MS, Maher DM, Khan S, Jaggi M, Chauhan SC: Current status and implications of microRNAs in ovarian cancer diagnosis and therapy. J Ovarian Res 2012; 5: 44

332. Nishimura M, Jung EJ, Shah MY, Lu C, Spizzo R, Shimizu M, Han HD, Ivan C, Rossi S, Zhang X, Nicoloso MS, Wu SY, Almeida MI, Bottsford-Miller J, Pecot CV, Zand B, Matsuo K, Shahzad MM, Jennings NB, Rodriguez-Aguayo C, Lopez-Berestein G, Sood AK, Calin GA: Therapeutic synergy between microRNA and siRNA in ovarian cancer treatment. Cancer Discov 2013; 3: 1302-15

333. Satoh J, Kino Y, Niida S: MicroRNA-Seq Data Analysis Pipeline to Identify Blood Biomarkers for Alzheimer's Disease from Public Data. Biomark Insights 2015; 10: 21-31

334. Burgos K, Malenica I, Metpally R, Courtright A, Rakela B, Beach T, Shill H, Adler C, Sabbagh M, Villa S, Tembe W, Craig D, Van Keuren-Jensen K: Profiles of extracellular miRNA in cerebrospinal fluid and serum from patients with Alzheimer's and Parkinson's diseases correlate with disease status and features of pathology. PLoS One 2014; 9: e94839

335. Keklikoglou I, Koerner C, Schmidt C, Zhang JD, Heckmann D, Shavinskaya A, Allgayer H, Guckel B, Fehm T, Schneeweiss A, Sahin O, Wiemann S, Tschulena U: MicroRNA-520/373 family functions as a tumor suppressor in estrogen receptor negative breast cancer by targeting NF-kappaB and TGF-beta signaling pathways. Oncogene 2012; 31: 4150-63

336. Nazarov PV, Reinsbach SE, Muller A, Nicot N, Philippidou D, Vallar L, Kreis S: Interplay of microRNAs, transcription factors and target genes: linking dynamic expression changes to function. Nucleic Acids Res 2013; 41: 2817-31

337. Horbelt D, Denkis A, Knaus P: A portrait of Transforming Growth Factor beta superfamily signalling: Background matters. Int J Biochem Cell Biol 2012; 44: 469-74

338. Wang W, Huang XR, Li AG, Liu F, Li JH, Truong LD, Wang XJ, Lan HY: Signaling mechanism of TGF-beta1 in prevention of renal inflammation: role of Smad7. J Am Soc Nephrol 2005; 16: 1371-83

339. Ji RR, Gereau RWt, Malcangio M, Strichartz GR: MAP kinase and pain. Brain Res Rev 2009; 60: 135-48

340. Wang S, Zhang L, Ma Y, Chen L, Tian Y, Mao J, Martyn JJ: Nociceptive behavior following hindpaw burn injury in young rats: response to systemic morphine. Pain Med 2011; 12: 87-98

341. Kulyte A, Belarbi Y, Lorente-Cebrian S, Bambace C, Arner E, Daub CO, Heden P, Ryden M, Mejhert N, Arner P: Additive effects of microRNAs and transcription factors on CCL2 production in human white adipose tissue. Diabetes 2014; 63: 1248-58

342. Itokazu T, Hayano Y, Takahashi R, Yamashita T: Involvement of Wnt/beta-catenin signaling in the development of neuropathic pain. Neurosci Res 2014; 79: 34-40

343. Simonetti M, Agarwal N, Stosser S, Bali KK, Karaulanov E, Kamble R, Pospisilova B, Kurejova M, Birchmeier W, Niehrs C, Heppenstall P, Kuner R: Wnt-Fzd Signaling Sensitizes Peripheral Sensory Neurons via Distinct Noncanonical Pathways. Neuron 2014; 83: 104-21

344. Mortensen MM, Hoyer S, Orntoft TF, Sorensen KD, Dyrskjot L, Borre M: High miR-449b expression in prostate cancer is associated with biochemical recurrence after radical prostatectomy. BMC Cancer 2014; 14: 859

345. Liu X, Feng J, Tang L, Liao L, Xu Q, Zhu S: The regulation and function of miR-21-FOXO3a-miR-34b/c signaling in breast cancer. Int J Mol Sci 2015; 16: 3148-62

346. Lan H, Lu H, Wang X, Jin H: MicroRNAs as Potential Biomarkers in Cancer: Opportunities and Challenges. Biomed Res Int 2015; 2015: 125094

347. Peng Y, Liu YM, Li LC, Wang LL, Wu XL: microRNA-503 inhibits gastric cancer cell growth and epithelial-to-mesenchymal transition. Oncol Lett 2014; 7: 1233-1238

348. Ye F, Tang H, Liu Q, Xie X, Wu M, Liu X, Chen B, Xie X: miR-200b as a prognostic factor in breast cancer targets multiple members of RAB family. J Transl Med 2014; 12: 17

349. Chen XN, Wang KF, Xu ZQ, Li SJ, Liu Q, Fu DH, Wang X, Wu B: MiR-133b regulates bladder cancer cell proliferation and apoptosis by targeting Bcl-w and Akt1. Cancer Cell Int 2014; 14: 70

350. Sanchez Freire V, Burkhard FC, Kessler TM, Kuhn A, Draeger A, Monastyrskaya K: MicroRNAs may mediate the down-regulation of neurokinin-1 receptor in chronic bladder pain syndrome. Am J Pathol 2010; 176: 288-303

351. Douglas SD, Leeman SE: Neurokinin-1 receptor: functional significance in the immune system in reference to selected infections and inflammation. Ann N Y Acad Sci 2011; 1217: 83-95

352. Shi L, Wang L, Wang B, Cretoiu SM, Wang Q, Wang X, Chen C: Regulatory mechanisms of betacellulin in CXCL8 production from lung cancer cells. J Transl Med 2014; 12: 70

353. Dai DW, Xu Z, Chen X, Yuan L, Zhang AJ, Zhang PQ, Lu YM, Deng ZF: Distinct roles of neuregulin in different models of neuropathic pain. Neurol Sci 2014; 35: 531-6

354. Zhu N, Zhang D, Chen S, Liu X, Lin L, Huang X, Guo Z, Liu J, Wang Y, Yuan W, Qin Y: Endothelial enriched microRNAs regulate angiotensin II-induced endothelial inflammation and migration. Atherosclerosis 2011; 215: 286-93

355. Jaggi AS, Singh N: Intrathecal delivery of farnesyl thiosalicylic acid and GW 5074 attenuates hyperalgesia and allodynia in chronic constriction injury-induced neuropathic pain in rats. Neurol Sci 2013; 34: 297-304

356. Quinn SR, Mangan NE, Caffrey BE, Gantier MP, Williams BR, Hertzog PJ, McCoy CE, O'Neill LA: The role of Ets2 transcription factor in the induction of microRNA-155 (miR-155) by lipopolysaccharide and its targeting by interleukin-10. J Biol Chem 2014; 289: 4316-25

357. Ji H, Houghton AM, Mariani TJ, Perera S, Kim CB, Padera R, Tonon G, McNamara K, Marconcini LA, Hezel A, El-Bardeesy N, Bronson RT, Sugarbaker D, Maser RS, Shapiro SD, Wong KK: K-ras activation generates an inflammatory response in lung tumors. Oncogene 2006; 25: 2105-12

358. McCullough SD, Duncan KE, Swanton SM, Dailey LA, Diaz-Sanchez D, Devlin RB: Ozone Induces a Pro-inflammatory Response in Primary Human Bronchial Epithelial Cells Through MAP Kinase Activation Without NF-kappaB Activation. Am J Respir Cell Mol Biol 2014

359. Miyagawa S, Katsu Y, Watanabe H, Iguchi T: Estrogen-independent activation of erbBs signaling and estrogen receptor alpha in the mouse vagina exposed neonatally to diethylstilbestrol. Oncogene 2004; 23: 340-9

360. Kalet BT, Anglin SR, Handschy A, O'Donoghue LE, Halsey C, Chubb L, Korch C, Duval DL: Transcription factor Ets1 cooperates with estrogen receptor alpha to stimulate estradiol-dependent growth in breast cancer cells and tumors. PLoS One 2013; 8: e68815

361. Pratt MA, Satkunaratnam A, Novosad DM: Estrogen activates raf-1 kinase and induces expression of Egr-1 in MCF-7 breast cancer cells. Mol Cell Biochem 1998; 189: 119-25

362. Aupperlee MD, Zhao Y, Tan YS, Leipprandt JR, Bennett J, Haslam SZ, Schwartz RC: Epidermal growth factor receptor (EGFR) signaling is a key mediator of hormone-induced leukocyte infiltration in the pubertal female mammary gland. Endocrinology 2014; 155: 2301-13

363. Horii J, Hiraoka S, Kato J, Saito S, Harada K, Fujita H, Kaji E, Yamamoto K: Methylation of estrogen receptor 1 in colorectal adenomas is not age-dependent, but is correlated with K-ras mutation. Cancer Sci 2009; 100: 1005-11

364. Radovick S, Levine JE, Wolfe A: Estrogenic regulation of the GnRH neuron. Front Endocrinol (Lausanne) 2012; 3: 52

365. Zhu Y, Sullivan LL, Nair SS, Williams CC, Pandey AK, Marrero L, Vadlamudi RK, Jones FE: Coregulation of estrogen receptor by ERBB4/HER4 establishes a growth-promoting autocrine signal in breast tumor cells. Cancer Res 2006; 66: 7991-8

366. Tanabe E, Shibata A, Inoue S, Kitayoshi M, Fukushima N, Tsujiuchi T: Regulation of cell motile activity through the different induction of LPA receptors by estrogens in liver epithelial WB-F344 cells. Biochem Biophys Res Commun 2012; 428: 105-9

367. Practice Committee of the American Society for Reproductive M: Treatment of pelvic pain associated with endometriosis. Fertil Steril 2006; 86: S18-27

368. Bazin S, Lefebvre J, Fortier M, Brisson J, Brouillette F, Bujold E, Bouchard C: [Evaluation of an estrogen vaginal cream for the treatment of dyspareunia: a double-blind randomized trial]. J Obstet Gynaecol Can 2011; 33: 838-43

369. Haj-Ahmad TA, Abdalla MA, Haj-Ahmad Y: Potential Urinary miRNA Biomarker Candidates for the Accurate Detection of Prostate Cancer among Benign Prostatic Hyperplasia Patients. J Cancer 2014; 5: 182-91

370. Ito T, Sato F, Kan T, Cheng Y, David S, Agarwal R, Paun BC, Jin Z, Olaru AV, Hamilton JP, Selaru FM, Yang J, Matsumura N, Shimizu K, Abraham JM, Shimada Y, Mori Y, Meltzer SJ: Polo-like kinase 1 regulates cell proliferation and is targeted by miR-593* in esophageal cancer. Int J Cancer 2011; 129: 2134-46

371. Shigehara K, Yokomuro S, Ishibashi O, Mizuguchi Y, Arima Y, Kawahigashi Y, Kanda T, Akagi I, Tajiri T, Yoshida H, Takizawa T, Uchida E: Real-time PCR-based analysis of the human bile microRNAome identifies miR-9 as a potential diagnostic biomarker for biliary tract cancer. PLoS One 2011; 6: e23584

372. Pereira PM, Marques JP, Soares AR, Carreto L, Santos MA: MicroRNA expression variability in human cervical tissues. PLoS One 2010; 5: e11780

373. Ninio-Many L, Grossman H, Levi M, Zilber S, Tsarfaty I, Shomron N, Tuvar A, Chuderland D, Stemmer SM, Ben-Aharon I, Shalgi R: MicroRNA miR-125a-3p modulates molecular pathway of motility and migration in prostate cancer cells. Oncoscience 2014; 1: 250-61

374. Vetter G, Saumet A, Moes M, Vallar L, Le Bechec A, Laurini C, Sabbah M, Arar K, Theillet C, Lecellier CH, Friederich E: miR-661 expression in SNAI1-induced epithelial to mesenchymal transition contributes to breast cancer cell invasion by targeting Nectin-1 and StarD10 messengers. Oncogene 2010; 29: 4436-48

375. Lam WY, Yeung AC, Ngai KL, Li MS, To KF, Tsui SK, Chan PK: Effect of avian influenza A H5N1 infection on the expression of microRNA-141 in human respiratory epithelial cells. BMC Microbiol 2013; 13: 104

376. Dominguez F, Moreno-Moya JM, Lozoya T, Romero A, Martinez S, Monterde M, Gurrea M, Ferri B, Nunez MJ, Simon C, Pellicer A: Embryonic miRNA profiles of normal and ectopic pregnancies. PLoS One 2014; 9: e102185

377. Hewagama A, Gorelik G, Patel D, Liyanarachchi P, McCune WJ, Somers E, Gonzalez-Rivera T, Michigan Lupus C, Strickland F, Richardson B: Overexpression of X-linked genes in T cells from women with lupus. J Autoimmun 2013; 41: 60-71

378. Dong Y, Li P, Ni Y, Zhao J, Liu Z: Decreased microRNA-125a-3p contributes to upregulation of p38 MAPK in rat trigeminal ganglions with orofacial inflammatory pain. PLoS One 2014; 9: e111594

379. Frantz C, Stewart KM, Weaver VM: The extracellular matrix at a glance. J Cell Sci 2010; 123: 4195-200

380. Dina OA, Parada CA, Yeh J, Chen X, McCarter GC, Levine JD: Integrin signaling in inflammatory and neuropathic pain in the rat. Eur J Neurosci 2004; 19: 634-42

381. Kim B, Feldman EL: Insulin resistance in the nervous system. Trends Endocrinol Metab 2012; 23: 133-41

382. Calura E, Cagnin S, Raffaello A, Laveder P, Lanfranchi G, Romualdi C: Meta-analysis of expression signatures of muscle atrophy: gene interaction networks in early and late stages. BMC Genomics 2008; 9: 630

383. Schwander M, Shirasaki R, Pfaff SL, Muller U: Beta1 integrins in muscle, but not in motor neurons, are required for skeletal muscle innervation. J Neurosci 2004; 24: 8181-91

384. Dimitriadis G, Mitrou P, Lambadiari V, Maratou E, Raptis SA: Insulin effects in muscle and adipose tissue. Diabetes Res Clin Pract 2011; 93 Suppl 1: S52-9

385. Luo J, Sobkiw CL, Hirshman MF, Logsdon MN, Li TQ, Goodyear LJ, Cantley LC: Loss of class IA PI3K signaling in muscle leads to impaired muscle growth, insulin response, and hyperlipidemia. Cell Metab 2006; 3: 355-66

386. Stenzel D, Lundkvist A, Sauvaget D, Busse M, Graupera M, van der Flier A, Wijelath ES, Murray J, Sobel M, Costell M, Takahashi S, Fassler R, Yamaguchi Y, Gutmann DH, Hynes RO, Gerhardt H: Integrin-dependent and -independent functions of astrocytic fibronectin in retinal angiogenesis. Development 2011; 138: 4451-63

387. Lei WL, Xing SG, Deng CY, Ju XC, Jiang XY, Luo ZG: Laminin/beta1 integrin signal triggers axon formation by promoting microtubule assembly and stabilization. Cell Res 2012; 22: 954-72

388. Saito F, Moore SA, Barresi R, Henry MD, Messing A, Ross-Barta SE, Cohn RD, Williamson RA, Sluka KA, Sherman DL, Brophy PJ, Schmelzer JD, Low PA, Wrabetz L, Feltri ML, Campbell KP: Unique role of dystroglycan in peripheral nerve myelination, nodal structure, and sodium channel stabilization. Neuron 2003; 38: 747-58

389. Zhou Q, Verne GN: miRNA-based therapies for the irritable bowel syndrome. Expert Opin Biol Ther 2011; 11: 991-5

390. Zhou Q, Souba WW, Croce CM, Verne GN: MicroRNA-29a regulates intestinal membrane permeability in patients with irritable bowel syndrome. Gut 2010; 59: 775-84

391. Goetsch MF: Unprovoked vestibular burning in late estrogen-deprived menopause: a case series. J Low Genit Tract Dis 2012; 16: 442-6

392. Jeon Y, Kim Y, Shim B, Yoon H, Park Y, Shim B, Jeong W, Lee D: A retrospective study of the management of vulvodynia. Korean J Urol 2013; 54: 48-52

393. Ventolini G, Barhan S, Duke J: Vulvodynia, a step-wise therapeutic prospective cohort study. J Obstet Gynaecol 2009; 29: 648-50

394. Harris G, Horowitz B, Borgida A: Evaluation of gabapentin in the treatment of generalized vulvodynia, unprovoked. J Reprod Med 2007; 52: 103-6

395. Ciszek BP, Khan AA, Dang H, Slade GD, Smith S, Bair E, Maixner W, Zolnoun D, Nackley AG: MicroRNA expression profiles differentiate chronic pain condition subtypes. Transl Res 2015

396. Hartung JE, Ciszek BP, Nackley AG: beta- and beta-adrenergic receptors drive COMT-dependent pain by increasing production of nitric oxide and cytokines. Pain 2014

397. Borsook D, Becerra L, Hargreaves R: Biomarkers for chronic pain and analgesia. Part 2: how, where, and what to look for using functional imaging. Discov Med 2011; 11: 209-19

398. Ikeda Y, Tanji E, Makino N, Kawata S, Furukawa T: MicroRNAs associated with mitogen-activated protein kinase in human pancreatic cancer. Mol Cancer Res 2012; 10: 259-69

399. Busch-Dienstfertig M, Gonzalez-Rodriguez S: IL-4, JAK-STAT signaling, and pain. JAKSTAT 2013; 2: e27638

400. Luo W, Sehgal A: Regulation of circadian behavioral output via a MicroRNA-JAK/STAT circuit. Cell 2012; 148: 765-79

401. Castaldi A, Zaglia T, Di Mauro V, Carullo P, Viggiani G, Borile G, Di Stefano B, Schiattarella GG, Gualazzi MG, Elia L, Stirparo GG, Colorito ML, Pironti G, Kunderfranco P, Esposito G, Bang ML, Mongillo M, Condorelli G, Catalucci D: MicroRNA-133 modulates the beta1-adrenergic receptor transduction cascade. Circ Res 2014; 115: 273-83

402. Zhang J, Banerjee B: Role of MicroRNA in Visceral Pain. J Neurogastroenterol Motil 2015; 21: 159-71

403. Mogil JS, Chanda ML: The case for the inclusion of female subjects in basic science studies of pain. Pain 2005; 117: 1-5

404. Baran-Gale J, Fannin EE, Kurtz CL, Sethupathy P: Beta cell 5'-shifted isomiRs are candidate regulatory hubs in type 2 diabetes. PLoS One 2013; 8: e73240

405. Langmead B, Trapnell C, Pop M, Salzberg SL: Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. Genome Biol 2009; 10: R25

406. Rumble SM, Lacroute P, Dalca AV, Fiume M, Sidow A, Brudno M: SHRiMP: accurate mapping of short color-space reads. PLoS Comput Biol 2009; 5: e1000386

407. Ebhardt HA, Fedynak A, Fahlman RP: Naturally occurring variations in sequence length creates microRNA isoforms that differ in argonaute effector complex specificity. Silence 2010; 1: 12

408. Kim VN, Han J, Siomi MC: Biogenesis of small RNAs in animals. Nat Rev Mol Cell Biol 2009; 10: 126-39

409. Lee LW, Zhang S, Etheridge A, Ma L, Martin D, Galas D, Wang K: Complexity of the microRNA repertoire revealed by next-generation sequencing. RNA 2010; 16: 2170-80

410. Selitsky SR, Baran-Gale J, Honda M, Yamane D, Masaki T, Fannin EE, Guerra B, Shirasaki T, Shimakami T, Kaneko S, Lanford RE, Lemon SM, Sethupathy P: Small tRNA-derived RNAs are increased and more abundant than microRNAs in chronic hepatitis B and C. Sci Rep 2015; 5: 7675